IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	: Annapragada, et al.	Examiner	: Perreira, Melissa Jean
Application No.	: 10/830,190	Group Art	: 1618
Filing Date	: 21 April 2004	Docket No.	: 27428-4
Confirmation No.	: 7714		
Title	: Compositions and Methods for Enhancing Contrast in Imaging		

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United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir/Madam:

This Appeal Brief is submitted pursuant to the Notice of Appeal filed March 3, 2008 in the above-identified application. This is an appeal from the decision of the Examiner mailed November 21, 2007. This Appeal Brief is filed on April 15, 2008, which is within two months of the filing date of the Notice of Appeal, along with a Certificate of Electronic Filing. Therefore, this Appeal Brief is timely filed. This Appeal Brief is accompanied by the fee set forth in 37 C.F.R. § 41.20(b)(2).

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TABLE OF AUTHORITIES

Cases

Statutes

Other Authorities

I. REAL PARTY IN INTEREST

The real party in interest is Marval BioSciences, Inc., the assignee of record.

II. RELATED APPEALS AND INTERFERENCES

None.

III. STATUS OF CLAIMS

A. The status of the claims in the application:

1. Claims 1-4, 6-11, 25, and 27-33 stand rejected under 35 U.S.C. 103(a) as being obvious over Torchilin et al. (Biochim. Biophys. Acta 1996, 1279, 75-83) ("Torchilin") in view of Payne et al. (U.S. Patent No. 4,744,989) ("Payne") and further in view of Sachse et al. (Invest. Radiol. 1997, 32, 44-50) ("Sachse") or Leike et al. (Invest. Radiol. 2001, 36, 303-308) ("Leike").

2. No basis for rejection was given for claim 26, although Form PTOL-326 of the Non-Final Office Action mailed November 21, 2007 (hereinafter the "Non-Final Office Action," Section IX, Evidence Appendix, Tab A) indicates that claim 26 stands rejected.

B. The claims on appeal:

Claims 1-4, 6-11, and 25-33 are on appeal (see Section VIII, Claims Appendix).

IV. STATUS OF AMENDMENTS

Appellant amended claims 1-4, 6-11, 25-28, 30, and 31 after the mailing of the Non-Final Office Action, but prior to the filing of the Notice of Appeal. The amendments were made to present the rejected claims in better form for consideration on appeal, pursuant to 37 C.F.R. § 1.116(b)(2). As of the date of filing of this Appeal Brief, Appellant had not received an advisory

action or any other communication from the Office regarding the Office's intention to refuse to enter the amendments. Appellant also checked the Office's Patent Application Information Retrieval (PAIR) system on the date of filing of this Appeal Brief, and found no indication that the Office intends to refuse to enter the proposed amendments. Thus, Appellant understands that all of the proposed amendments have been entered.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The subject application contains two independent claims, namely claims 1 and 25, both of which are involved in the appeal.

Claim 1 recites a composition for enhancing contrast of one or more areas of a subject for X-ray imaging when administered to the subject. (Specification, at p. 17, ¶ 0052, Section IX, Evidence Appendix, Tab B). The composition comprises liposomes, each liposome encapsulating one or more nonradioactive contrast-enhancing agents. (Id., at pp. 3-4, ¶ 0019). Each liposome comprises: cholesterol, at least one phospholipid, and at least one phospholipid which is derivatized with a polymer chain. (Id., at p. 11, ¶ 0034). The average diameter of the liposomes is less than 150 nanometers. (Id., at p. 15, ¶ 0045).

Claim 25 also recites a composition for enhancing contrast of one or more areas of a subject for X-ray imaging when administered to the subject. (Id., at p. 17, ¶ 0052). The composition comprises liposomes, each liposome comprising: at least one first lipid or phospholipid; at least one second lipid or phospholipid which is derivatized with one or more polymers; and at least one sterically bulky excipient capable of stabilizing the liposomes. (Id., at p. 11, ¶ 0034). The average diameter of the liposomes is less than 150 nanometers. (Id., at p. 15, ¶ 0045). Each liposome encapsulates at least one nonradioactive contrast enhancing agent. (Id., at pp. 3-4, ¶ 0019).

The size of the liposomes (< 150 nm mean diameter) leads to increased circulation times in the blood. Additionally, the use of polymer-derivatized lipids prevents interaction between the liposomes and blood plasma components, the plasma components playing a role in the uptake

of liposomes by cells of the blood and removal of the liposomes from the blood. (Id., at p. 10, ¶ 0029).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Whether claims 1-4, 6-11, 25, and 27-33 are unpatentable under 35 U.S.C. 103(a) as being obvious over Torchilin (Section IX, Evidence Appendix, Tab C) in view of Payne (Section IX, Evidence Appendix, Tab D) and further in view of Sachse (Section IX, Evidence Appendix, Tab E) or Leike (Section IX, Evidence Appendix, Tab F).

B. Whether claim 26 may be rejected without providing any basis for the rejection.

VII. ARGUMENT

Claims 1-4, 6-11, 25, and 27-33 stand rejected under 35 U.S.C. 103(a). The Office failed to provide a basis for the rejection of claim 26. As set forth below, the Office has not met its burden to support these rejections and, accordingly, the rejections should be reversed and this application should be passed to allowance.

A. Claims 1-4, 6-11, 25, and 27-33 are not obvious over Torchilin in view of Payne and further in view of Sachse or Leike, and the rejections under 35 U.S.C. § 103(a) should be reversed.

Claims 1-4, 6-11, 25, and 27-33 stand rejected under 35 U.S.C. § 103(a). 35 U.S.C. § 103(a) provides, in pertinent part:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

The factual inquiries relevant to establishing obviousness under 35 U.S.C. § 103(a) are set forth in Graham v. John Deere Co., 383 U.S. 1 (1966):

(1) Determining the scope and contents of the art being cited.

(2) Ascertaining the differences between the referenced art and the claims at issue.

(3) Resolving the level of ordinary skill in the pertinent art.

(4) Considering objective evidence present in the application indicating obviousness or nonobviousness.

The Office, in its *Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in KSR International Co. v. Teleflex Inc.*, 72 Fed. Reg. 57526, 57529 (Oct. 10, 2007), stated: "It can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." See also May 3, 2007 PTO Memorandum, at p. 2 ("[I]n formulating a rejection under 35 U.S.C. § 103(a) based upon a combination of prior art elements, it remains necessary to identify the reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed.").

1. Scope and contents of the cited art.

a. Torchilin.

Torchilin addresses biodistribution of liposomes in acutely damaged tissues with broken-down vascular and cell membrane barriers. The primary goal of Torchilin is to characterize immunoliposomes, specifically those that have antibodies to myosin on their exterior. (Torchilin, at Abstact, p. 76). To accomplish this goal, Torchilin seeks to determine antibody-liposome immunoreactivity by direct binding of radiolabeled antibody-liposome and antibody-liposome-polymer conjugates. (Id., at p. 77). The Torchilin liposomes are prepared by mixing egg yolk phosphatidylcholine (PC), cholesterol, and dioleoylphosphatidylethanolamine-polyethylene glycol (PEG-PE). (Id.). Torchilin does not teach or suggest liposomes having a nonradioactive contrast enhancing agent encapsulated therein. Indeed, Torchilin does not teach a contrast enhancing agent at all. Rather, Torchilin affirmatively teaches a radioactive tracer—In^{111}, externally trans-chelated to DTPA. This is because Torchilin is not directed to enhancing contrast of one or more areas of a subject for X-ray imaging.

b. Payne.

Payne "relates to a method for preparing particulate water-soluble carrier materials coated with thin films of liposome components . . . ." (Payne, at col. 1, l. 11-13). Payne teaches the use of "adjuvants," such as cholesterol. (Id., at col. 7, l. 5). Payne also teaches the use of "biologically active compounds," such as iodinated contrast agent. (Id., at col. 6, l. 1-41).

Payne purports to achieve the preparation of a "final liposome product of desired size, such as a mean diameter of within the range of from about 25 nm to about 12 μm" (Id., at col. 4, l. 54-58). However, Payne teaches that the composition of the liposomes used is a key determinant of their size. (Id., at col. 4, l. 59 – col. 5, l. 3). Important in relation to this principle is the fact that Payne does not teach or even suggest a liposome having lipids or phospholipids which are derivatized with a polymer.

The examples of Payne almost exclusively demonstrate liposome sizes of several microns. See, e.g., Examples 1 (5.3 μm) (Id., at col. 8, l. 56), 2 (2.5 μm) (Id., at col. 9, l. 15), 3 (5.3 μm) (Id., at col. 9, l. 40), 4 (2.5 μm) (Id., at col. 9, l. 67), and 7 (1.8 μm, 2.0 μm, 3.1 μm, and 4.25 μm) (Id., at col. 12, l. 33 and col. 13, l. 22, 60, 62). In Example 7, Payne refers to liposomes (composed of DMPC/DMPG/AmB) with "mean sizes of 100 to 150 nm" (Id., at col. 13, l. 24), but those liposomes clearly: (1) are not polymer derivatized; (2) do not contain cholesterol; and (3) do not contain non-radioactive contrast enhancing agent.

c. Sachse.

Sachse is directed to PEG-coated iopromide-carrying liposomes. (Sachse, at Abstract). Specifically, Sachse teaches the coating of liposomes consisting of soy PC (SPC), cholesterol, and soy phosphatidylglycerol (SPG), and having a mean vesicle size of 132 nm, with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG (DSPE-PEG), to yield liposomes having a mean diameter of 204 nm. (Id., at p. 3, para. 8). Sachse does not teach liposomes having polymer-chain derivatized phospholipids wherein the liposomes have an average diameter of less than 150 nm. Rather, Sachse specifically teaches that the inclusion of DSPE-PEG leads to a "drastic increase in vesicle size." (Id.).

d. Leike.

Leike is directed to iopromide-carrying liposomes that are potentially useful as CT blood-pool agents. (Leike, at p. 303). Leike does not teach phospholipids derivatized with polymer chains. (Id., at pp. 303, 305) ("Iopromide liposomes composed of SPC, cholesterol, and SPG in a molar ratio of 6:3:1 were prepared . . . ."). In fact, Leike specifically states, "In the present study, tolerance, elimination, and diagnostic properties of **unmodified** (conventional) iopromide-carrying blood-pool liposomes were studied." (Id., at p. 306) (emphasis added). Leike does not teach liposomes having an average diameter of less than 150 nm. (Id., at pp. 305, 307) ("The resulting mean diameter amount to 201 nm . . . .") ("mean diameter ≈ 200 nm").

2. The differences between the cited art and the claims at issue.

Amended claim 1 calls for liposomes which encapsulate one or more nonradioactive contrast-enhancing agents, the liposomes comprising cholesterol, at least one phospholipid, and at least one phospholipid which is derivatized with a polymer chain, wherein the liposomes are less than 150 nanometers in average diameter.

Amended claim 25 calls for liposomes comprising at least one first lipid or phospholipid; at least one second lipid or phospholipid which is derivatized with one or more polymers; and at least one sterically bulky excipient capable of stabilizing the liposomes; wherein the liposomes are less than 150 nanometers in average diameter, and wherein the liposomes encapsulate at least one nonradioactive contrast enhancing agent.

Both of amended claims 1 and 25 are directed to compositions "for enhancing contrast of one or more areas of a subject for X-ray imaging when administered to the subject."

As its principal reference, the Office cites Torchilin. As set forth above, Torchilin does not teach liposomes having a nonradioactive contrast enhancing agent encapsulated therein. Indeed, Torchilin does not teach a contrast enhancing agent at all. Torchilin is not silent on a tracing component, however. Rather, Torchilin affirmatively teaches a radioactive tracer—In^{111}, externally trans-chelated to DTPA. This is because Torchilin is not directed to enhancing

contrast of one or more areas of a subject for X-ray imaging. Instead, Torchilin is directed to determining antibody-liposome immunoreactivity by direct binding of radiolabeled antibody-liposome and antibody-liposome-polymer conjugates. A prima facie case of obviousness requires that "[a] prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention." M.P.E.P. § 2141.02(VI). Simply put, a person having ordinary skill in the art at the relevant time would have had no motivation to combine Torchilin (radiolabeled antibody liposomes) with Payne (non-radioactive contrast enhancing agent), Sachse (same), or Leike (same).

As a secondary reference, the Office cites Payne. Again, Payne discloses that the composition of the liposomes used is a key determinant of their size. While Payne purports to achieve the preparation of a "final liposome product of desired size, such as a mean diameter of within the range of from about 25 nm to about 12 μm," (Payne, at col. 4, l. 54-58), Payne does not teach or even suggest a liposome having lipids or phospholipids which are derivatized with a polymer. Thus, Payne simply cannot be properly cited for teaching the manipulation of the size of liposomes comprising a lipid or phospholipid derivatized with a polymer and encapsulating a nonradioactive contrast enhancing agent.

Moreover, notwithstanding Payne's broad (and unsupported) statement regarding mean diameter, the examples of Payne almost exclusively demonstrate sizes of several microns. See, e.g., Examples 1 (5.3 μm), 2 (2.5 μm), 3 (5.3 μm), 4 (2.5 μm), and 7 (1.8 μm, 2.0 μm, 3.1 μm, and 4.25 μm). As noted above, in Example 7, Payne refers to liposomes (composed of DMPC/DMPG/AmB) with "mean sizes of 100 to 150 nm" (Id., at col. 13, l. 24), but those liposomes clearly: (1) are not polymer derivatized; (2) do not contain cholesterol; and (3) do not contain non-radioactive contrast enhancing agent. In short, the Office's implication that Payne teaches the ready manipulation of the size of relevant liposomes is incorrect and, in fact, flies in the face of Payne's own teaching regarding the relationship between the composition of a liposome and its size.

With respect to its dubious combination of Torchilin and Payne, the Office states: "The disclosures [of Torchilin and Payne] are drawn to the same products (liposomes) and the encapsulation of the contrast agents of Payne et al. into the liposomes of Torchilin et al. will have predictable results, as there are multiple factors for controlling the size of the liposomes." (Non-Final Office Action, at ¶ 13, p. 6).

Appellant respectfully suggests that the Office has grossly oversimplified the cited art and the subject application. Indeed, following the teachings of Payne, in combination with Torchilin, will not lead to the claimed invention.

Moreover, the Office impermissibly ignores the plain teachings of Sachse, which post-dates Torchilin and Payne, when the Office dismisses the inventiveness of achieving the claimed compositions, having a mean diameter of less than 150 nm, as the result of "routine experimentation" (Id.):

> Subsequently, surface-modification [of Iopromide-carrying liposomes] was performed by simple mixing with the respective PEG-derivative overnight. In the case of DSPE-PEG this procedure was accompanied by a **drastic increase in vesicle size. Thus, the resulting mean diameter amounted to 204 nm compared to 132 for the unmodified** . . . .

(Sachse, at p. 3, para. 8) (emphasis added). Based on the teachings of Sachse (who is a person having extraordinary skill in the art), the achievement of the claimed liposome compositions—i.e., having both nonradioactive contrast enhancing agent and polymer-derivatized lipids or phospholipids, and having a mean diameter of less than 150 nm, is not obvious. To the contrary, Sachse represents irrefutable evidence demonstrating a lack of expectation of success.

Moreover, the substitution of the non-radioactive contrast enhancing agents of Payne for the radioactive tracer of Torchilin would clearly render Torchilin inoperable (i.e., to determine antibody-liposome immunoreactivity by direct binding of radiolabeled antibody-liposome and antibody-liposome-polymer conjugates). (Torchilin, at p. 77). See In re Fitch, 972 F.2d 1260, 1265 n.12 (Fed. Cir. 1992) ("A proposed modification [is] inappropriate for an obviousness

inquiry when the modification render[s] the prior art reference inoperable for its intended purpose.").

The Office further contends that: "It would be obvious to try/substitute the different lipids taught by Sachse et al. or Leike et al. for the lipids of the iodine agent containing/encapsulating liposomes of the combined disclosures of Payne et al. and Torchilin et al. as they are advantageous and suited for CT blood-pool imaging with iodinated contrast agents." (Non-Final Office Action, at ¶ 17, p. 7).

First, Leike does not teach phospholipids derivatized with polymer chains: "In the present study, tolerance, elimination, and diagnostic properties of **unmodified** (conventional) iopromide-carrying blood-pool liposomes were studied." (Leike, at p. 306) (emphasis added). Leike also does not teach liposomes having an average diameter of less than 150 nm. (Leike, p. 305) (201 nm). Thus, substitution of the lipids taught by Leike would not achieve the claimed invention.

Second, the liposomes of Sachse, as described more fully above, also have a size greater than 200 nm, and, thus, their substitution would also not achieve the claimed invention. As described above, the combination of the disclosures of Payne and Torchilin, of which Sachse had the benefit, already has failed to prevent a "dramatic increase in size" upon Sachse's PEGylation of Sachse's 132 nm liposomes. (Sachse, at p. 3, para. 8).

Finally, when positing what "would be obvious to try" regarding the various liposomes of the cited art for CT blood-pool imaging, the teachings representative of the state of the art in the relevant time period must be considered. (72 Fed. Reg. 57526, 57527). For example, the Office must consider U.S. Patent No. 6,217,849 issued to Tournier et al., which post-dates Torchilin, Sachse, and Payne (Payne was cited against Tournier). Tournier teaches vesicles in the 200 nm to 1 μm range, with an average diameter of 400 nm. (Tournier, col. 4, l. 60-67). Tournier clearly teaches away from the use of the small liposomes of the subject application:

> The use of tiny liposome vesicles of the kind proposed in EP-A-0 442 962 for the delivery of drugs (in the order of 50 nm or less) are [sic] therefore **unpractical for blood-pool imaging**. Much **the same applies to** the proposals of Gabison et al. in Biochim. Et Biophys. Acta 1103 (1992) 94-100 and I.A.J.M. Bakker-Woudenberg et al. ibid 318-326 directed to **liposomes with an average size between 0.07 μm and 0.1 μm** and prolonged residence times in the blood.

(Tournier, at col. 3, l. 14-22) (emphasis added). Tournier also teaches away from the use of polymer-derivatized liposomes. (Id., at col. 3, l. 30-35):

> [T]he production of liposomes with the "stealth factors" is rather cumbersome. In addition, **"stealth factored" liposomes** are known to have very low entrapment capacity and while such liposomes may be suitable to carry specific drugs, and therefore useful in therapy, they **are almost useless in imaging**.

(Id., col. 3, lines 30-35) (emphasis added). It should be noted that liposomes containing polymer-derivatized lipids, such as those of the subject application, are often referred to in the art as "stealth factored" liposomes.

Thus, Tournier, another person having extraordinary skill in the art, who had the benefit of the teachings of Torchilin, Sachse, and Payne, specifically taught that stealth factors (i.e., polymer-derivatized lipids and phospholipids) are "almost useless in imaging," and that small liposomes are "unpractical for blood pool imaging."

The teachings of Tournier are not counterbalanced by the Office's citation of Leike in the Non-Final Office Action (Non-Final Office Action, at para. 17, p. 7), as Leike expressly excluded polymer-derivatized liposomes and liposomes having a mean diameter of less than 200 nm. (Leike, at pp. 305, 306). Leike (2001) had the benefit of Tournier. Jens U. Leike is also an author of the Sachse publication, which was published prior to Tournier. Thus, if anything, Leike's express exclusion of polymer-derivatized lipids and small liposomes in his second publication should be viewed as an adoption of Tournier's teachings against the use of stealth factors and small liposomes in imaging, further demonstrating an expectation of a lack of success at the relevant time period, i.e., when the invention was made. 35 U.S.C. § 103(a).

For the foregoing reasons, claims 1-4, 6-11, 25, and 27-33 are not obvious over Torchilin in view of Payne and further in view of Sachse or Leike, and the rejections under 35 U.S.C. § 103(a) should be reversed.

B. The Office's rejection of claim 26 without providing a basis is improper and should be reversed.

35 U.S.C. § 132(a) provides as follows regarding the Office's duty to inform an applicant of the results of the Office's examination:

> Whenever, on examination, any claim for a patent is rejected, or any objection or requirement made, the Director shall notify the applicant thereof, **stating the reasons for such rejection**, or objection or requirement, together with such information and references as may be useful in judging the propriety of continuing the prosecution of his application.

(Emphasis added). 37 C.F.R. § 1.104(a)(2) expands on this statutory requirement:

> The applicant . . . will be notified of the examiner's action. **The reasons for any adverse action** or any objection or requirement **will be stated in an Office action** and such information or references will be given as may be useful in aiding the applicant . . . to judge the propriety of continuing the prosecution.

(Emphasis added). Clearly, the Office is statutorily required to state the reasons for a rejection. The Office failed to provide any basis for its rejection of claim 26, although Form PTOL-326 of the Non-Final Office Action indicates that claim 26 stands rejected. Thus, the rejection of claim 26 is improper and should be reversed.

C. Conclusion.

For the foregoing reasons, Appellant respectfully asserts that the case is now in a condition for allowance. While no additional fees are believed due, the Commissioner is hereby authorized to charge any additional fees, or credit any overpayment, to Deposit Account No. 02-2051, referencing Attorney Docket No. 27428-4.

Respectfully submitted,

Dated: April 15, 2008

By: /s/Benjamen E. Kern
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VIII. CLAIMS APPENDIX

1. (Previously amended) A composition for enhancing contrast of one or more areas of a subject for X-ray imaging when administered to the subject, the composition comprising:

liposomes, each liposome encapsulating one or more nonradioactive contrast-enhancing agents, and each liposome comprising: cholesterol, at least one phospholipid, and at least one phospholipid which is derivatized with a polymer chain,

wherein the average diameter of the liposomes is less than 150 nanometers.

2. (Previously amended) The composition of claim 1, wherein the X-ray imaging is computed tomography.

3. (Previously amended) The composition of claim 1, wherein the nonradioactive contrast-enhancing agents are selected from at least one of: iodinated ionic compounds, iodinated nonionic compounds, and mixtures therof.

4. (Previously amended) The composition of claim 3, wherein a suspension of the liposomes has a concentration of at least 30 milligrams of iodine per milliliter of the suspension.

5. (Cancelled).

6. (Previously amended) The composition of claim 1, wherein the average diameter of the liposomes is less than 120 nanometers.

7. (Previously amended) The composition of claim 1, wherein the composition is capable of being administered to the bloodstream of the subject.

8. (Previously amended) The composition of claim 7, wherein the composition provides an enhanced contrast that remains detectable at least 30 minutes after administration.

9. (Previously amended) The composition of claim 7, wherein the composition provides an enhanced contrast of at least 50 Hounsfield units in at least part of at least one of a vasculature and an organ of the subject.

10. (Previously amended) The composition of claim 1, wherein the liposomes are PEGylated liposomes.

11. (Previously amended) The composition of claim 1, wherein the liposomes are targeted liposomes.

12.-24. (Cancelled).

25. (Previously amended) A composition for enhancing contrast of one or more areas of a subject for X-ray imaging when administered to the subject, the composition comprising liposomes, each liposome comprising:

at least one first lipid or phospholipid;

at least one second lipid or phospholipid which is derivatized with one or more polymers; and

at least one sterically bulky excipient capable of stabilizing the liposomes;

wherein the average diameter of the liposomes is less than 150 nanometers, and wherein each liposome encapsulates at least one nonradioactive contrast enhancing agent.

26. (Previously amended) The composition of claim 25, wherein the at least one first lipid or phospholipid comprises 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC).

27. (Previously amended) The composition of claim 25, wherein the at least one second lipid or phospholipid which is derivatized with one or more polymers comprises [N-(carbonylmethoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphatidylcholine] (DSPE-MPEG2000).

28. (Previously amended) The composition of claim 25, wherein the at least one sterically bulky excipient is selected from at least one of: sterols, fatty alcohols, fatty acids, and mixtures thereof.

29. (Previously amended) The composition of claim 25, wherein the at least one sterically bulky excipient is cholesterol.

30. (Previously amended) The composition of claim 25, wherein the liposomes are not autoclaved.

31. (Previously amended) The composition of claim 25, wherein the liposomes are contained in a suspension medium, at least some of the contrast enhancing agent that has not been encapsulated by the liposomes having been removed from the suspension medium.

32. (Previously presented) The composition of claim 25, wherein the at least one first lipid or phospholipid is present in the amount of about 55 to about 75 mol %; the at least one second lipid or phospholipid which is derivatized with one or more polymers is present in the amount of about 1 to about 20 mol %; and the at least one sterically bulky excipient is present in the amount of about 25 to about 40 mol %.

33. (Previously amended) The composition of claim 32, wherein the at least one first lipid or phospholipid is hydrogenated soy phosphatidylcholine; the at least one second lipid or phospholipid which is derivatized with one or more polymers is [N-(carbonylmethoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphatidylcholine] (DSPE-MPEG2000); and the at least one sterically bulky excipient is cholesterol.

IX. EVIDENCE APPENDIX

Attached hereto are true and correct copies of the following evidence entered by the Office and relied upon by Appellant in this Appeal Brief. Citations to specific portions of these documents may be found in Appellant's Argument in section VII of this Appeal Brief.

TAB A: November 21, 2007 Non-Final Office Action

TAB B: Specification

TAB C: Torchilin et al. (Biochim. Biophys. Acta 1996, 1279, 75-83)

TAB D: U.S. Patent No. 4,744,989 issued to Payne et al.

TAB E: Sachse et al. (Invest. Radiol. 1997, 32, 44-50)

TAB F: Leike et al. (Invest. Radiol. 2001, 36, 303-308)

X. RELATED PROCEEDINGS APPENDIX

None.

TAB A

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO
10/830,190	04/21/2004	Ananth Annapragada	27428-4	7714

21130 7590 11/21/2007

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EXAMINER	
PERREIRA, MELISSA JEAN	
ART UNIT	**PAPER NUMBER**
1618	

MAIL DATE	DELIVERY MODE
11/21/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/830,190	ANNAPRAGADA ET AL	
	Examiner	Art Unit	
	Melissa Perreira	1618	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *18 September 2007*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-4,6-11 and 25-33* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-4,6-11 and 25-33* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *21 April 2004* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 8/24/07 has been entered.

Claims and Previous Rejections Status

2. Claims 1-4,6-11 and 25-33 are pending in the application.

3. The rejection under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement is withdrawn.

4. The rejection under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is withdrawn.

5. The rejection under 35 U.S.C. 103(a) as being unpatentable over Klaveness et al. (US 5,676,928) or Tournier et al. (US 6,217,849B1) in view of Torchilin et al. (*Biochim. Biophys. Acta* **1996**, *1279*, 75-83) is withdrawn.

6. The declaration under 37 CFR 1.132 filed 9/18/07 is acknowledged but is not relevant in view of the new grounds of rejection.

New Grounds of Rejection

The rejection under 35 U.S.C. 103(a) as being unpatentable over Leike et al. (*Invest. Radiol.* **2001**, *36*, 303-308) in view of Torchilin et al. (*Biochim. Biophys. Acta* **1996**, *1279*, 75-83) or Sachse et al. (*Invest. Radiol.* **1997**, *32*, 44-50) as stated in the office action mailed 8/24/07 has been modified due to the amendment.

Claim Rejections - 35 USC § 103

7. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

8. Claims 1-4,6-11,25 and 27-33 are rejected under 35 U.S.C. 103(a) as being unpatentable over Torchilin et al. (*Biochim. Biophys. Acta* **1996**, *1279*, 75-83) in view of Payne et al. (US 4,744,989) and further in view of Sachse et al. (*Invest. Radiol.* **1997**, *32*, 44-50; pages provided are numbered 1-8) or Leike et al. (*Invest. Radiol.* **2001**, *36*, 303-308).

9. Torchilin et al. (*Biochim. Biophys. Acta* **1996**, *1279*, 75-83) discloses a PEG and/or antibody substituted liposome which are long-circulating and target-specific (p76, paragraphs 2 and 3). The blood circulation time of the liposomes are improved by coating the surface with PEG by decreasing their opsonization and recognition by the liver (p76, paragraph 2). The targeting of liposomes to infracted myocardium is possible

since normal myocardial cells do not permit extracellular macromolecules, such as antimyosin antibody, to traverse the cell membrane but necrotic cardiomyocytes with disrupted membranes cannot prevent the antibody from interacting with myosin (p76, paragraph 1). The liposomes are prepared by mixing PC, cholesterol and a PEG-PE (p77, paragraph 3). The liposomes of the disclosure include small liposomes of size 120-150 nm. According to applicants declaration filed 9/18/07, the recitation of "incorporated" (liposomes are radioactively labeled with the radioactive element via liposome-incorporated chelating agent DTPA (p77, paragraph 4) is defined as the external attachment of the radiolabeled contrast agent outside of the liposome. Therefore, prior to incorporation of the radioactively labeled contrast agent the targeted, pegylated liposomes of the disclosure include small liposomes of size 120-150 nm (p77, preparation of liposomes). Torchilin et al. does not disclose the encapsulation of an iodinated contrast agents.

10. Payne et al. (US 4,744,989) discloses liposomes prepared from a combination of lipids (column 5, lines 64-65) and adjuvants, such as cholesterol where the mean size of the liposome can be controlled to suit the particular medicament, such as an iodinated contrast agent (column 6, lines 11-30) to be carried by the liposome (column 3, lines 31-33; column 4, lines 33-36; column 4, lines 59-62). The liposomes of the disclosure have a mean size from about 100 nm to 6 microns (column 4, lines 57-58). The size may be affected by the amount of phospholipids, the pH and hydration medium (column 5, lines 1-9). The method of preparing the liposomes includes subsequent removal of the unencapsulated material (column 6, lines 60-61).

11. Sachse et al. (*Invest. Radiol.* **1997**, *32*, 44-50; pages provided are numbered 1-8) teaches of Iopromide-containing liposomes for enhancing CT imaging. The liposomes contain soy phosphatidylcholine (SPC), cholesterol, soy phosphatidylglycerol (SPG) (6:3:1 molar ratio) and 5 mol% DPSE-PEG2000 which are administered intravenously into a rat tail vein at a dose of 250mg I/kg (p3, paragraph 4) and show prolonged blood circulation with CT density differences above 70 HU (abstract; p2, paragraph 1). The CT blood pool imaging in a rabbit with DSPE-PEG liposomes show approximately 71ΔHU after 45 min (p5, paragraph 4; fig 6A-6D). Sachse et al. also discloses that the PEGylated lipid derivatives in the liposome membrane provides for potent increase in circulation times (p1, paragraph 2) as they avoid the mononuclear phagocytic system (MPS) and target to non-MPS organs.

12. Leike et al. (*Invest. Radiol.* **2001**, *36*, 303-308) discloses a computed tomography enhancing iodinated liposome composition containing soy phosphatidylcholine (SPC), cholesterol and soy phosphatidylglycerol (SPG) (p303, last paragraph). The contrast enhancing liposomal agents have a mean diameter of 201 nm are used for prolonged blood-pool opacification upon intravenous injection of 300mg I/kg (p305, paragraphs 3 and 8; p306, fig 2) which encompass the compositions for enhancing contrast of the instant claims. The contrast enhancing iodinated liposome compositions of the disclosure are observed immediately after administration up to 60 min with a mean peak enhancement of in the aorta of approximately 90ΔHU (p305, last paragraph; p306, first paragraph).

13. At the time of the invention it would have been obvious to one skilled in the art to prepare targeted-pegylated liposomes of the size 120nm-150 nm (Torchilin et al.) and utilize/try them for the encapsulation of the iodinated contrast agents of Payne et al. as the liposomes of Payne et al. may also be 100 nm in size. Torchilin et al. teaches that the blood circulation time for PEG-LL (large liposomes) is less than that for PEG-SL (small liposomes) (p81, paragraph 1). The disclosures are drawn to the same products (liposomes) and the encapsulation of the contrast agents of Payne et al. into the liposomes of Torchilin et al. will have predictable results, as there are multiple factors for controlling the size of the liposomes. The substitution of different lipids as taught by Sachse et al. or Leike et al. for the lipids of Torchilin et al. is advantageous as they are well suited for CT blood-pool imaging with iodinated contrast agents (Leike et al. p303, paragraph 1). In the case of small liposomes, Torchilin et al. (p80, small liposomes) teaches that grafting of PEG to the liposome surface sharply increases the liposomal circulation time due to the interaction of the PEG with plasma proteins (p80, small liposomes). Furthermore, it is obvious to vary and/or optimize the amount of (compound) provided in the composition, according to the guidance provided by (reference), to provide a composition having the desired properties such as the desired (ratios, concentrations, percentages, etc.). It is noted that "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." In re Aller, 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955).

Response to Arguments

14. Applicant asserts that Torchilin et al. teaches a preparation of liposomes where the attachment of the radioactive tracer is on the outside of the liposome as opposed to encapsulated within.

15. Torchilin et al. does teach that prior to incorporation of the radioactively labeled contrast agent the targeted, pegylated liposomes of the disclosure include small liposomes of size 120-150 nm (p77, preparation of liposomes). The reference of Torchilin et al. was used to teach of the preparation of these small liposomes and not encapsulation of the contrast enhancing agents.

16. Applicant asserts that there in no motivation to combine Leike et al. and Torchilin et al. as the liposomes of Torchilin et al. may very well render Leike et al. compositions inoperable, and vice-versa.

17. It would be obvious to try/substitute the different lipids taught by Sachse et al. or Leike et al. for the lipids of the iodine agent containing/encapsulating liposomes of the combined disclosures of Payne et al. and Torchilin et al. as they are advantageous and suited for CT blood-pool imaging with iodinated contrast agents (Leike et al. p303, paragraph 1). Applicant assertion that the liposomes of Torchilin et al. **may very well render** Leike et al. compositions inoperable, and vice-versa is opinion and conjecture and does not eliminate the fact that it would be obvious to try the lipids of Sachse et al. or Leike et al. for their advantageous characteristics.

Conclusion

No claims are allowed at this time.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Melissa Perreira whose telephone number is 571-272-1354. The examiner can normally be reached on 9am-5pm M-F.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Mike Hartley can be reached on 571-272-0616. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

MP
November 13, 2007

MICHAEL G. HARTLEY
SUPERVISORY PATENT EXAMINER

Notice of References Cited	Application/Control No. 10/830,190	Applicant(s)/Patent Under Reexamination ANNAPRAGADA ET AL	
	Examiner Melissa Perreira	Art Unit 1618	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-4,744,989 A	05-1988	Payne et al	424/490
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action (See MPEP § 707 05(a))
Dates In MM-YYYY format are publication dates. Classifications may be US or foreign

TAB B

U.S. Non-Provisional Patent Application

Attorney Docket No.: 27428-4

Title:

COMPOSITIONS AND METHODS FOR ENHANCING CONTRAST IN IMAGING

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COMPOSITIONS AND METHODS FOR ENHANCING CONTRAST IN IMAGING

Background

[0001] Some medical X-ray imaging techniques provide for detecting variations in contrast of regions of interest of a subject, including different organs, tissues, cells and the like. In order to increase the contrast of regions of interest, some of the imaging techniques may provide for administration of contrast-enhancing agents to the subject. The contrast-enhancing agents may accentuate existing differences in contrast between different areas of interest, or may produce differences in contrast where such differences do not exist without use of the agents.

[0002] There have been advancements in medical X-ray imaging, specifically relating to the instruments or machines used to detect the differences in contrast. Such advancements include increases in the speed of such instruments, increases in the resolution of such instruments, and the like. These advancements have provided, in part, for new medical imaging methods. One such method, whole-body imaging, may provide information on the vasculature of the entire body of a subject.

[0003] Compared to advances in the instruments used for X-ray imaging, advances in contrast-enhancing agents have not been as forthcoming. Current contrast-enhancing agents for medical imaging using X-rays may have limitations for applications such as whole-body imaging due to, among other things, their rapid clearance from the body, prevalent extravasation, their renal toxicity and their inability to target specific areas of the body.

Brief Description Of The Drawings

[0004] In the accompanying drawings, which are incorporated in and constitute a part of the specification, embodiments of contrast-enhancing agent formulations, pharmaceutical compositions containing the formulations, methods for making the formulations and methods for using the formulations in imaging are illustrated which, together with the detailed description given below, serve to describe the example embodiments of formulations, compositions, methods, and so on. It will be appreciated that the embodiments illustrated in the drawings are shown for the purpose of illustration and not for limitation. It will be appreciated that changes, modifications and deviations from the embodiments illustrated in

the drawings may be made without departing from the spirit and scope of the invention, as disclosed below.

[0005] **Figure 1** illustrates an example method **100** of preparing liposomes containing or associated with contrast-enhancing agents;

[0006] **Figure 2** illustrates another example method **200** of preparing liposomes containing or associated with contrast-enhancing agents;

[0007] **Figure 3** shows an example *in vitro* stability test **300** of one embodiment of a liposomal iohexol formulation when dialyzed with PBS at 4°C. The total iodine amount is 30 mg iodine;

[0008] **Figure 4** shows an example *in vitro* plasma stability test **400** of one embodiment of a liposomal iohexol formulation when dialyzed against PBS at 37°C. The total iodine content is 28 mg iodine;

[0009] **Figure 5** shows example time-attenuation curves **500** of various regions of interest at different post-injection times after intravenous administration of one embodiment of a liposomal iohexol formulation (injection to 2.2 kg rabbit vein at a dose of 475 mg I/kg) given in two incremental injections;

[0010] **Figure 6** shows example pre- and post-enhancement computed tomography (CT) images **600** of one embodiment of liposomal iohexol: 2.2 kg rabbit with 34.8 mg/ml iodine IV injection. Left Panels **605, 615**: pre-contrast; Right Panels **610, 620**: 2 hours 18 minutes post injection. Upper panels **605, 610** are images taken at the level of the liver. Lower panels **615, 620** are images taken at mid heart level;

[0011] **Figure 7** shows example volume-rendered CT images of a rabbit torso **700**. Left panel **705**: right lateral view before contrast injection; Right panel **710**: right lateral view 2 hours 18 minutes after injection of 475 mg I/kg of one embodiment of a liposomal iohexol formulation. Note the enhanced vascular bed seen in the right panel **715**;

[0012] **Figure 8** shows example volume-rendered CT images of an *in vivo* rabbit heart **800** imaged before **805** and at multiple time sequences post injection **810, 815, 820, 825, 830** of one embodiment of liposomal iohexol. All volume-rendering parameters and display parameters were held constant across time points;

[0013] **Figure 9** shows an example of a thick-slab rendering of ultra-high resolution CT scan (24 line pair per cm) of post-mortem rabbit (no cardiac motion) **900**. Rabbit was sacrificed 3.5 hours after the second injection of one embodiment of liposomal iohexol. Images were reconstructed to fit a 1,024 X 1,024 matrix with a 0.5-cm field of view; and

[0014] **Figure 10** shows an example image of the left coronary artery of the rabbit under high magnification **1000**.

Detailed Description

Definitions

[0015] Definitions of selected terms or phrases are contained immediately following, and throughout the disclosure. The definitions include examples of various embodiments and/or forms of components that fall within the scope of a term and that may be used for implementation. The examples are not intended to be limiting and other embodiments may be implemented. Both singular and plural forms of all terms fall within each meaning.

[0016] "X-ray imaging," as used herein, generally refers to any of a number of procedures using a source producing X-rays. Examples of X-ray imaging include computed tomography and the like.

[0017] "Computed tomography" or "CT" or "CAT," as used herein, generally refers to procedures using a rotating X-ray instrument or machine to produce X-ray radiation and direct it through areas of a subject as the instrument rotates. The radiation that is not absorbed by the subject generally is detected and recorded. Generally, the data are sent to a computer which creates detailed cross-sectional images, or slices, of organs and body parts based on differential absorption of X-rays by different areas of the subject.

[0018] "Whole body imaging," as used herein, generally refers to methodologies for obtaining images, using CT for example, of the entire body of a subject. In one type of whole body imaging, the entire vasculature system may be examined. Generally, imaging where the vasculature system is examined is called "blood pool imaging."

Description

[0019] This application describes example compositions comprising liposomes which contain or are associated with one or more contrast-enhancing agents. In one embodiment,

the liposomes contain or are associated with relatively high concentrations of contrast-enhancing agents. In one example, the liposomes contain one or more contrast-enhancing agents for X-ray imaging, CT for example. In one example, the contrast-enhancing agents are not radioactive.

[0020] In one embodiment, the liposomes have one or more hydrophilic polymers attached to or associated with the liposomes. In one example, the hydrophilic polymers are attached to or associated with the surface of the liposomes. When administered to a subject, the liposomes may provide increased contrast in the body of a subject. In one example, the increased contrast lasts for an extended period of time.

[0021] This application also describes example pharmaceutical compositions that contain the liposomes and contrast-enhancing agents, and example methods of making the compositions of liposomes containing contrast-enhancing agents. The application also describes example methods of using the compositions in X-ray imaging.

Contrast-Enhancing Agents

[0022] "Contrast-enhancing agent," as used herein, generally refers to a substance that affects the attenuation, or the loss of intensity or power, of radiation as it passes through and interacts with a medium. It will be appreciated that contrast-enhancing agents may increase or decrease the attenuation. Generally, the contrast-enhancing agents referred to herein increase the attenuation of radiation. In one embodiment, the contrast-enhancing agents described herein are contrast-enhancing agents for X-ray imaging. In one embodiment, the contrast-enhancing agents are used for CT. In one embodiment, the contrast-enhancing agents used herein are nonradioactive. In one embodiment, the contrast-enhancing agents contain iodine.

[0023] The contrast-enhancing agents may be classified in various ways. In one classification, for example, iodinated contrast-enhancing agents may be water soluble (e.g., monoiodinated pyridine derivatives, di-iodinated pyridine derivatives, tri-iodinated benzene ring compounds, and the like), water-insoluble (e.g., propyliodone and the like) or oily (e.g., iodine in poppy seed oil, ethyl esters of iodinated fatty acids of poppy seed oil containing iodine, and the like).

[0024] In one example, a grouping of iodinated contrast-enhancing agents are water soluble. Present water soluble iodinated contrast-enhancing agents are generally derivatives of tri-iodinated benzoic acid. These compounds may have one or more benzene rings. Such compounds may be ionic or nonionic. Example ionic compounds include acetrizoate, diatrizoate, iodamide, ioglicate, iothalamate, ioxithalamate, metrizoate, sodium meglumine ioxaglate and others. Example, nonionic compounds include metrizamide, iohexol, iopamidol, iopentol, iopromide, ioversol, iotrolan, iodixanol and others.

Liposomes

[0025] "Liposomes," as used herein, generally refer to spherical or roughly spherical particles containing an internal cavity. The walls of liposomes generally are comprised of a bilayer of lipids, particularly phospholipids. There are numerous lipids and phospholipids that can be used to make liposomes. For example, commonly used are amphipathic lipids having hydrophobic and polar head group moieties, and which can form spontaneously into bilayer vesicles in water, as exemplified by phospholipids, or are stably incorporated into lipid bilayers, with its hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and its polar head group moiety oriented toward the exterior, polar surface of the membrane.

[0026] As used herein, example "phospholipids" include but are not limited to phosphatidic acid (PA) and phosphatidylglycerol (PG), phosphatidylcholine (PC), egg phosphatidylcholine (EPC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). The vesicle-forming lipids of this type may be lipids having two hydrocarbon chains, typically acyl chains, and a polar head group. Included in this class are the phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), and sphingomyelin (SM), and others. These phospholipids may be fully saturated or partially saturated. They may be naturally occurring or synthetic. In another example, other lipids that can be included in the liposomes are glycolipids.

[0027] The phospholipids used in the example liposomes described herein may be those where the two hydrocarbon chains are between about 14-24 carbon atoms in length, and have varying degrees of unsaturation. Some examples of such phospholipids are given below, in **Table 1**. Although the phospholipids listed below may be used, alone or in combination with

other phospholipids, the list is not intended to be complete. Other phospholipids not listed may also be used.

Table 1. Phospholipids

1-Myristoyl-2-Palmitoyl-*sn*-Glycero-3-Phosphocholine

1-Myristoyl-2-Stearoyl-*sn*-Glycero-3-Phosphocholine

1-Myristoyl-2-Palmitoyl-*sn*-Glycero-3-Phosphocholine

1-Myristoyl-2-Stearoyl-*sn*-Glycero-3-Phosphocholine

1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphate (POPA)

1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine

1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphoethanolamine (POPE)

1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (POPG)

1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-L-Serine] (POPS)

1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphate

1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine

1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphoethanolamine

1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1-Palmitoyl-2-Arachidonoyl-*sn*-Glycero-3-Phosphate

1-Palmitoyl-2-Arachidonoyl-*sn*-Glycero-3-Phosphocholine

1-Palmitoyl-2-Arachidonoyl-*sn*-Glycero-3-Phosphoethanolamine

1-Palmitoyl-2-Arachidonoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1-Palmitoyl-2-Arachidonoyl-*sn*-Glycero-3-[Phosph-L-Serine]

1-Palmitoyl-2-Docosahexaenoyl-*sn*-Glycero-3-Phosphate

1-Palmitoyl-2-Docosahexaenoyl-*sn*-Glycero-3-Phosphocholine

1-Palmitoyl-2-Docosahexaenoyl-*sn*-Glycero-3-Phosphoethanolamine

1-Palmitoyl-2-Docosahexaenoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1-Palmitoyl-2-Docosahexaenoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1-Stearoyl-2-Myristoyl-*sn*-Glycero-3-Phosphocholine

1-Stearoyl-2-Palmitoyl-*sn*-Glycero-3-Phosphocholine

1-Stearoyl-2-Oleoyl-*sn*-Glycero-3-Phosphate

1-Stearoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine

1-Stearoyl-2-Oleoyl-*sn*-Glycero-3-Phosphoethanolamine

1-Stearoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol]

1-Stearoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphate

1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine

1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphoethanolamine

1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1-Stearoyl-2-Arachidonoyl-*sn*-Glycero-3-Phosphate

1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine

1-Stearoyl-2-Arachidonoyl-*sn*-Glycero-3-Phosphoethanolamine

1-Stearoyl-2-Arachidonoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1-Stearoyl-2-Arachidonoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1-Stearoyl-2-Docosahexaenoyl-*sn*-Glycero-3-Phosphate

1-Stearoyl-2-Docosahexaenoyl-*sn*-Glycero-3-Phosphocholine

1-Stearoyl-2-Docosahexaenoyl-*sn*-Glycero-3-Phosphoethanolamine

1-Stearoyl-2-Docosahexaenoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1-Stearoyl-2-Docosahexaenoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1-Oleoyl-2-Myristoyl-*sn*-Glycero-3-Phosphocholine

1-Oleoyl-2-Palmitoyl-*sn*-Glycero-3-Phosphocholine

1-Oleoyl-2-Stearoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dimyristoyl-*sn*-Glycero-3-Phosphate (DMPA)

1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC)

1,2-Dimyristoyl-*sn*-Glycero-3-Phosphoethanolamine (DMPE)

1,2-Dimyristoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DMPG)

1,2-Dimyristoyl-*sn*-Glycero-3-[Phospho-L-Serine] (DMPS)

1,2-Dipentadecanoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate (DPPA)

1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC)

1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine (DPPE)

1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DPPG)

1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-L-Serine) (DPPS)

1,2-Diphytanoyl-*sn*-Glycero-3-Phosphate

1,2-Diphytanoyl-*sn*-Glycero-3-Phosphocholine

1,2-Diphytanoyl-*sn*-Glycero-3-Phosphoethanolamine

1,2-Diphytanoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1,2-Diphytanoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1,2-Diheptadecanoyl-*sn*-Glycero-3-Phosphocholine

1,2-Distearoyl-*sn*-Glycero-3-Phosphate (DSPA)

1,2-Distearoyl-*sn*-Glycero-3-Phosphocholine (DSPC)

1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine (DSPE)

1,2-Distearoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DSPG)

1,2-Distearoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1,2-Dibromostearoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dinonadecanoyl-*sn*-Glycero-3-Phosphocholine

1,2-Diarachidoyl-*sn*-Glycero-3-Phosphocholine

1,2-Diheneicosanoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dibehenoyl-*sn*-Glycero-3-Phosphocholine

1,2-Ditricosanoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dilignoceroyl-*sn*-Glycero-3-Phosphocholine

1,2-Dimyristoleoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dimyristelaidoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dipalmitoleoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dipalmitelaidoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dipalmitoleoyl-*sn*-Glycero-3-Phosphoethanolamine

1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC)

1,2-Dioleoyl-*sn*-Glycero-3-Phosphate (DOPA)

1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC)

1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine (DOPE)

1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DOPG)

1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-L-Serine] (DOPS)

1,2-Dielaidoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dielaidoyl-*sn*-Glycero-3-Phosphoethanolamine

1,2-Dielaidoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1,2-Dilinoleoyl-*sn*-Glycero-3-Phosphate

1,2-Dilinoleoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dilinoleoyl-*sn*-Glycero-3-Phosphoethanolamine

1,2-Dilinoleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1,2-Dilinoleoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1,2-Dilinolenoyl-*sn*-Glycero-3-Phosphocholine

1,2-Dilinolenoyl-*sn*-Glycero-3-Phosphoethanolamine

1,2-Dilinolenoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1,2-Dieicosenoyl-*sn*-Glycero-3-Phosphocholine

1,2-Diarachidonoyl-*sn*-Glycero-3-Phosphate

1,2-Diarachidonoyl-*sn*-Glycero-3-Phosphocholine

1,2-Diarachidonoyl-*sn*-Glycero-3-Phosphoethanolamine

1,2-Diarachidonoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1,2-Diarachidonoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1,2-Dierucoyl-*sn*-Glycero-3-Phosphocholine

1,2-Didocosahexaenoyl-*sn*-Glycero-3-Phosphate

1,2-Didocosahexaenoyl-*sn*-Glycero-3-Phosphocholine

1,2-Didocosahexaenoyl-*sn*-Glycero-3-Phosphoethanolamine

1,2-Docosahexaenoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

1,2-Didocosahexaenoyl-*sn*-Glycero-3-[Phospho-L-Serine]

1,2-Dinervonoyl-*sn*-Glycero-3-Phosphocholine

[0028] The liposome composition may be formulated to include amounts of fatty alcohols, fatty acids, and/or cholesterol esters or other pharmaceutically acceptable excipients. For example, the liposomes may include lipids that can stabilize a vesicle or liposome composed predominantly of phospholipids. For example, cholesterol between about 25 to 40 mole percent may be used.

[0029] In one embodiment, the type of liposomes used are "sterically stabilized liposomes." Sterically stabilized liposomes generally include a surface that contains or is coated with flexible water soluble (hydrophilic) polymer chains. Such polymer chains may prevent interaction between the liposomes and blood plasma components, the plasma components playing a role in uptake of liposomes by cells of the blood and removal of the liposomes from the blood. Sterically stabilized liposomes may avoid uptake by the organs of the mononuclear phagocyte system, primarily the liver and spleen (reticulendothelial system or RES). Such sterically stabilized liposomes may also be called "long circulating liposomes."

[0030] Sterically stabilized liposomes may contain lipids or phospholipids which are derivatized with a polymer chain. The lipids or phospholipids which may be used generally are any of those described above. One exemplary phospholipid is phosphatidylethanolamine (PE) with a reactive amino group which is convenient for coupling to the activated polymers. An exemplary PE is distearyl PE (DSPE).

[0031] Examples of polymers which may be suitable for use in sterically stabilized liposomes include the hydrophilic polymers polyvinylpyrrolidone, polymethyloxazoline,

polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide, polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses, such as hydroxymethylcellulose or hydroxyethylcellulose. Polylysine may be used. Lipid-polymer conjugates containing these polymers attached to a suitable lipid, such as PE, may be used. Other example polymers may be used.

[0032] In one embodiment, the polymer in the derivatized lipid or phospholipid is polyethylene glycol (PEG). The PEG may have any of a variety of molecular weights. In one example, the PEG chain has a molecular weight between about 1,000-10,000 daltons. Once a liposome is formed, the PEG chains generally provide a surface coating of hydrophilic chains sufficient to extend the blood circulation time of the liposomes in the absence of such a coating. Such liposomes may be called "PEGylated liposomes." PEGylated liposomes include so-called STEALTH® liposomes, provided by ALZA Corporation.

[0033] PEGylated liposomes may also include liposomes with PEG on their surface, where the PEG may be released from the liposome at some time after administration of the liposomes to a subject. In one embodiment, there may be one or more bonds or linkages attaching the PEG, or other hydrophilic polymer, to the liposome surface and/or lipid molecules comprising the liposome surface. In one embodiment, the bonds or linkages may be cleaved, providing for separation of the PEG from the liposome. For example, PEG may be attached to a lipid by one or more disulfide bonds. The disulfide bonds may be cleaved by free thiol, releasing the PEG from the liposome. Other types of cleavable links or bonds may be used to attach the polymers to the liposomes. Other types of agents or compounds may be used to cleave the bonds or linkages.

[0034] In one example, the liposomes used have a composition of between about 60 and 75 mole % of one or more of the phospholipids with carbon chains between about 14-24 in length, as described above. A fraction of these phospholipids may be attached to one or more hydrophilic polymers such that between about 1 and 20 mole % of the liposome composition is phospholipid derivatized with polymer chains. In addition, the liposomes used may have between about 25 and 40 mole % cholesterol, or fatty alcohols, fatty acids, and/or other cholesterol esters or other pharmaceutically acceptable excipients, generally for the purpose of stabilizing the liposomes.

[0035] In another embodiment, the liposomes may have a molecule or molecules, commonly called a "ligand," accessible from the surface of the liposome, that specifically binds or attaches to, for example, one or more molecules or antigens. Such ligands may direct or target the liposomes to a specific cell or tissue and may bind to a molecule or antigen on or associated with the cell or tissue. The ligand may be an antibody or antibody fragment. The antibody may be a monoclonal antibody or fragment. Such liposomes may be of a type called "targeted liposomes."

[0036] In one example, targeted liposomes have lipids or phospholipids which have been modified for coupling antibody molecules to the liposome outer surface. These modified lipids may be of different types. The modified lipid may contain a spacer chain attached to the lipid. The spacer chain may be a hydrophilic polymer. The hydrophilic polymer may typically be end-functionalized for coupling antibody to its functionalized end. The functionalized end group may be a maleimide group, for selective coupling to antibody sulfhydryl groups. Other functionalized end groups may include bromoacetamide and disulfide groups for reaction with antibody sulfhydryl groups, activated ester and aldehyde groups for reaction with antibody amine groups. Hydrazide groups are reactive toward aldehydes, which may be generated on numerous biologically relevant compounds. Hydrazides may also be acylated by active esters or carbodiimide-activated carboxyl groups. Acyl azide groups reactive as acylating species may be easily obtained from hydrazides and permit the attachment of amino containing ligands.

[0037] In another embodiment, the phospholipid may be modified by a biotin molecule. To attach the antibody molecule to the biotinylated liposome surface, once the liposome is formed, the antibody molecule may also be modified with biotin and then incubated in the presence of the avidin. Biotinylated lipids, such as biotinylated PE, may be commercially available.

[0038] In another example, lipids may be modified by a substrate for use in binding a targeting molecule to a liposome surface. Typically, substrates, as exemplified with biotin, are relatively small, less than about 5,000 daltons for example, to allow their incorporation into multilamellar liposomes with a minimum of disruption of the lipid bilayer structures. The substrate may be one capable of binding irreversibly to a targeting molecule, to ensure that the targeting molecule remains bound to the liposomes over its lifetime in the bloodstream.

Preparation of Liposomes Containing Contrast-Enhancing Agents

[0039] The liposomes generally may be prepared by a variety of methods. Example methods include, but are not limited to, hydration of dried lipids, introduction of a volatile organic solution of lipids into an aqueous solution causing evaporation of the organic solution, and dialysis of an aqueous solution of lipids and detergents or surfactants to remove the detergents or surfactants. Additional methods are described in U.S. Pat. No. 5,049,389 by Radhakrishnan, entitled "Novel Liposome Composition for the Treatment of Interstitial Lung Diseases," the descriptions of which are incorporated herein in their entirety by reference.

[0040] Liposomes may contain or are associated with one or more contrast-enhancing agents. In one embodiment, the liposomes contain the contrast-enhancing agents. In the process of making liposomes, the contrast-enhancing agents can be added at any desired time. For example, contrast-enhancing agents may be associated with components of liposomes before liposomes are formed. Contrast-enhancing agents may be combined with liposome components at the time the liposomes are made. Contrast-enhancing agents may also be added after the liposomes are formed. Other methods of associating contrast-enhancing agents with liposomes may exist. Generally, contrast-enhancing agents which are hydrophilic in nature are located or associated with the internal cavity of the liposome particles. Contrast-enhancing agents which are lipophilic in nature generally are located or associated with the lipid bilayer of liposome particles. Generally, the contrast-enhancing agents herein are located or associated with the internal cavity of the liposome. The example liposomes may contain at least 30 mg iodine/milliliter (I/ml) of liposome suspension when iodinated contrast enhancing agents are used.

[0041] There are a variety of methods known in the art for loading the contrast-enhancing agents into the liposomes. For example, in passive loading, liposomes are formed in a solution of the contrast-enhancing agent to be used. Illustrated in **Figure 1** is one example method **100** that may be performed by selecting one or more contrast-enhancing agents to be used (block **105**) and forming liposomes in the presence of the one or more contrast-enhancing agents (block **110**). Generally, the methods described earlier for preparing liposomes may be used. Such methods include hydration of dried lipids, introduction of a volatile organic solution of lipids into an aqueous solution causing evaporation of the organic

solution, dialysis of an aqueous solution of lipids and detergents or surfactants to remove the detergents or surfactants, and others.

[0042] In active or remote loading, a variety of methods can be used. Generally, in these methods, the agent to be encapsulated by the liposomes (i.e., contrast-enhancing agents) is drawn or attracted into liposomes, or trapped or contained in liposomes as they are formed. In one example, **Figure 2** illustrates a method **200** that may be performed by forming liposomes (block **205**) and drawing one or more contrast-enhancing agents into the liposomes (block **210**). In one approach, an ion gradient may be established from the interior to the exterior of the liposome, and used to promote encapsulation of the desired active agent. In a common application of this technique, ammonium sulfate may be passively encapsulated in liposomes. Upon substitution of the external phase with a suitable (neutral pH) buffer, the ammonia may diffuse out of the liposome, while leaving behind the charged protons and sulfate ions. Neutral molecules of the active agent (i.e., contrast-enhancing agent) then may diffuse into the liposomes, down the concentration gradient, and are protonated, and possibly sulfated by the internal ions (see for example, Ceh and Lasic, 1997, J. Colloid Interface Sci., 185:9-18). In another embodiment of this approach, calcium acetate rather than ammonium sulfate may be the loading agent.

[0043] In another type of active loading, called pH based loading, a proton may be dissociated from the contrast-enhancing agents, causing the contrast-enhancing agents to enter into and remain in the liposomes (see Ceh and Lasic, 1997, J. Colloid Interface Sci., 185:9-18). In still another method, a chelating agent within the liposome may result in trapping of contrast-enhancing agents therein as well as further diffusion of contrast-enhancing agents into the liposomes (see Patent No. WO0023052 by Colbern, Working and Slater, entitled "Liposome-Entrapped Topoisomerase Inhibitors").

[0044] After the loading of contrast-enhancing agents into liposomes, steps may be used to remove contrast-enhancing agents that have not been loaded and are not associated with liposomes, or other impurities. Such steps may comprise techniques such as ion exchange, diafiltration, washing of the liposomes using ultracentrifugation, dialysis, and the like.

[0045] After liposomes are made, techniques for manipulating the liposomes may be used. For example, a preparation of liposomes made by standard techniques may vary in size and lamellarity (i.e., wall thickness) after it is made. Techniques such as subjecting the

liposomes to a high shearing force, extrusion of the liposomes through membranes, or sonication of the liposomes may be used either to select liposomes of a desired size or modify the liposomes such that they have a desired size. After manipulation of liposomes by these methods, the size distribution of the liposomes may be measured to ensure that liposomes of the desired size have been obtained. Techniques such as Fraunhofer diffraction and dynamic light scattering (DLS) may be used to measure the size distribution of the liposomes. Such techniques generally measure an equivalent spherical diameter which, in the case of Fraunhofer diffraction, may be the diameter of a sphere with the same light scattering properties as the measured liposomes. In the case of DLS, equivalent spherical diameter may be the diameter of a sphere with the same diffusion coefficient as the measured liposomes. Generally, the example liposomes have an average diameter of less than 150 nm. Example preparations of liposomes may have an average diameter of approximately 120 nm or less. Example preparations of liposomes may have an average diameter of approximately 100 nm or less. It will be appreciated that other sizes can be used.

[0046] In one embodiment, a nano-scale liposomal formulation carrying over 30 mg of iohexol per ml of liposome can be formulated using passive loading. In this formulation, the lipid composition of the bilayer has been adjusted as described below to allow this amount of contrast-enhancing agent to be encapsulated. In one example, it has been found that by using pure DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine) of C16 chain length, with about 40 mole % cholesterol and 5 mole % mPEG-DSPE (N-(carbonylmethoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine) (the polyethylene glycol-conjugated lipid that confers long circulating properties), the encapsulation of active molecules inside the liposomes may be increased by 20% over what is possible using hydrogenated Soy PC (HSPC), a mixture of C16 and C18 lipids, or pure DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) of C18 chain length. Using a formulation of 55 mole % DPPC, 40 mole % cholesterol and 5 mole % mPEG-DSPE and an iohexol solution of 350 mg I/ml, an overall concentration of over 30 mg I/ml has been achieved, with an average liposomal diameter of 100.6 ± 3 nm, as determined by DLS.

Pharmaceutical Compositions And Administration To Subjects

[0047] The liposomes containing or associated with one or more contrast-enhancing agents may be part of a pharmaceutical composition suitable for administration to a subject.

The compositions generally are administered using a route that delivers the composition to an area of interest. In one example, the compositions of contrast-enhancing agents are administered parenterally to the subject, such as through intravenous, intraarterial, subcutaneous or other route of injection.

[0048] The formulation of the particular pharmaceutical composition generally will depend on the method by which the composition is administered to a patient. It will be appreciated that the pharmaceutical compositions can include salt, buffering agents, preservatives, other vehicles and, optionally, other agents. Compositions suitable for parenteral administration may comprise a sterile, pyrogen-free, aqueous or oleaginous preparation which is generally isotonic with the blood of the subject. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent. Among acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride or other salt, dextrose, phosphate buffered saline and the like, or combinations thereof.

[0049] The pharmaceutical compositions used may also contain stabilizers, preservatives, buffers, antioxidants, or other additives. In addition, sterile, fixed oils may be employed as a solvent or suspending medium. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulations suitable for the administrations may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa. The pharmaceutical compositions may conveniently be presented in unit dosage form.

[0050] Parenteral administration contemplates the use of a syringe, catheter or similar device, which delivers the pharmaceutical composition to a site. Delivery may result, at least initially, in the pharmaceutical composition being systemically distributed throughout the circulatory system of the subject.

[0051] Generally, the pharmaceutical compositions may be administered to the subject at a point in time before the imaging of the subject is performed. The amount of the pharmaceutical compositions administered preferably results in increased contrast of one or more tissues of the subject. Ultimately, the attending physician or technician generally will decide the amount of pharmaceutical composition to administer to the subject. Generally, the

increase in contrast can be any level above what is present without use of the contrast-enhancing agents in the pharmaceutical compositions. Example increases in contrast of at least 50 HU, at least 100 HU or more, to one or more organ systems, including the vasculature, may be obtained.

Applications

[0052] The compositions of liposomes containing contrast-enhancing agents or pharmaceutical compositions thereof, when administered to a subject, may maintain a level of contrast-enhancing agent in the blood and/or organs of a subject that results in an increased contrast and is detectable by X-ray imaging techniques. The increase in contrast may be detectable for an extended period of time. Depending on the particular application, the compositions described herein may have half lives in the circulation of from minutes to hours, to even days. In one example, half lives in the circulation of from 8 to 24 hours can be obtained. In one example, an administered composition provides an enhanced contrast that remains detectable at least 30 minutes after administration. In another example, an administered composition provides an enhanced contrast that remains detectable at least 5 minutes after administration. Many applications, including those in anatomic, functional and molecular imaging may be possible. For example, use of the compositions described herein may have applications in cardiology, oncology, neurology and other areas.

[0053] In one embodiment, blood pool imaging may be used to detect and, in some cases, quantify ischemia. For example, because injection of the pharmaceutical compositions generally alters the contrast of the entire vasculature, reduced blood flow as is present in ischemia may be detected. A variety of types of ischemia may be detected, including that causing ischemic bowel disease, pulmonary embolism, and types of ischemia that produce cardiomyopathy, and others. In other applications, aneurysms may also be detected.

[0054] In one embodiment, the compositions described herein may be used in cardiac imaging to detect, examine and/or assess stenosis, and the therapy or remediation of stenosis, as occurs in angioplasty, for example. The utility of such techniques may be enhanced through the use of contrast-enhancing agent preparations, such as those described herein.

[0055] In one embodiment, the compositions described herein may be used to detect myocardial microcirculatory insufficiencies. Myocardial microcirculation is known to

display signs of obstruction before the epicardial arteries show signs of obstruction. Therefore, detection of obstruction in the myocardial microcirculation may be an earlier detector of atherosclerosis in presymptomatic, at-risk patients, than conventional methods. The compositions described herein may facilitate detection of obstructions in the myocardial microcirculation.

[0056] In another embodiment, the compositions described herein may be used to detect and characterize a wide range of tumors and cancers. These applications may be facilitated by the property of sterically stabilized liposomes being present for extended periods of time in the circulation and to extravasate at regions where the vasculature is "leaky," such as in tumors, for example. The leakiness of the vasculature in tumors may be attributed to the high proportion of neovasculature, the result of continuing angiogenesis as the tumor grows in size. Upon encountering such leaky vasculature, liposomes may leave the circulation, driven with the extravasate fluid, by hydrostatic pressure. Such liposomes generally do not return to the circulation after extravasation since the pressure gradient opposes such return. Such methods can be used to detect both primary and metastatic tumors.

[0057] In other embodiments, the compositions may be used for "staging" or classification of tumors. Such applications may depend on, among other things, differences in the "leakiness" of the vasculature of a given tumor or cancer at different stages of progression.

[0058] In one embodiment, the compositions may be used in the area of monitoring and characterizing injury and healing of damaged spinal cords. In a typical spinal cord injury, as occurs in an automobile accident for example, there may also be damage to tissue surrounding the spinal cord. It is thought that the process of healing of the surrounding tissue may be deleterious to healing of the spinal cord. It is thought that formation of neovasculature in the surrounding tissue, as occurs in healing of the surrounding tissue, may inhibit healing of the spinal cord. It is thought that by inhibiting healing of the surrounding tissue, and the formation of neovasculature in the surrounding tissue, the spinal cord may heal. Subsequently, the surrounding tissue may heal. The compositions of contrast-enhancing agents described here may be useful for monitoring the healing and inhibition of healing of the tissue surrounding the spinal cord.

[0059] There may be a variety of other applications for the compositions described herein. For example, the compositions may be used in detection and monitoring of inflammation, reperfusion injuries, and the like.

[0060] Additionally, the liposomes which comprise the compositions of contrast-enhancing agents may be targeted to desired cells and tissues in the body of a subject by, for example, attaching antibodies to the surface of the liposomes. Such targeting may result in enhanced contrast to the targeted areas of the body.

[0061] The compositions of contrast-enhancing agents may have a relatively long residence time in the body, low extravasation, except in those areas of the vasculature that are leaky as described above, may be relatively nontoxic to the kidneys and may be used to target specific areas of the body. Additionally, the traditional osmolality related toxicity problems associated with ionic contrast-enhancing media generally are not an issue with the liposomal encapsulates since the high osmolality phase is interior to the liposomes and generally is not exposed to the blood.

Examples

Example 1. Preparation of PEGylated Liposomes Containing Iohexol

[0062] Example liposomal iohexol formulations can be produced as follows. Briefly, a lipid mixture (200 mM) of 1,2-Dipalmitoly-sn-glycero-3-phosphocholine (DPPC), cholesterol (chol) and N-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-MPEG2000), in a 55:40:5 molar ratio, was dissolved in ethanol at 65°C. The ethanol solution was then hydrated with iohexol (350 mg I/ml) for 1.5-2 hours. Liposomes were extruded on a 10 ml Lipex Thermoline extruder (Northern Lipids, Vancouver, British Columbia, Canada) with 5 passes through a 0.2 μm Nucleopore membrane (Waterman Inc., Newton Massachusetts) and 7 passes through a 0.1 μm Nucleopore membrane (Waterman Inc., Newton Massachusetts). Liposomes were then be dialyzed in a 300,000 molecular weight cutoff (MWCO) dialysis bag against phosphate buffer saline (PBS) overnight to remove the free iohexol.

[0063] The size of the resulting example liposomal iohexol formulations can be determined by dynamic light scattering (DLS) using a modified BI-90 goniometer, a JDS uniphase 532 nm laser, Hamamastu photomultiplier and Brookhaven DLS Software Version

3.16. The average diameter of the liposomal iohexol capsules was 100.6 nm (STD = 3.0 nm), which is in nano-scale range, as determined by DLS.

[0064] The iohexol concentrations of example liposomal iohexol formulations can be determined by measuring the absorption at 245 nm using a UV-Vis spectrophotometer. Equivalent iodine concentrations can then be calculated. In the example preparations, different lipid hydration times (1.5 hours and 2 hours) resulted in different iohexol loading concentrations (30 and 34.8 mg I/ml respectively). The 30 mg I/ml iohexol liposomal formulation was used in the *in vitro* stability tests described below, and the 34.8 mg I/ml iohexol liposomal formulation were used in the *in vivo* CT imaging experiment described below.

[0065] The osmolarity of liposomal iohexol formulation can be measured by, for example, Vapro® vapor pressure osmometer (Wescor Inc.). The osmolarity of the example iohexol formulations ranged between 305 to 315 mmol/kg.

Example 2. *In Vitro* Stability of PEGylated Liposomes Containing Iohexol

[0066] The *in vitro* stability of example liposomal iohexol formulations can be determined by measuring the leakage of iohexol from liposomal iohexol formulations both in PBS at 4°C and in plasma at 37°C. In the procedure, 1 ml of an example liposomal iohexol formulation was placed in a 300,000 MWCO dialysis bag and dialyzed against 250 ml PBS at 4°C. At each time point (0, 1, 2, 3, 8, 24 hours, and 3, 4, 5, 6, 8, 10, 18 days), 1 ml of the dialysate was removed for a UV absorption-based iohexol measurement. At least three data points were obtained at each time point. After measurement, samples were returned to the PBS to maintain constant volume.

[0067] To measure stability in plasma, the example liposomal iohexol formulations can be dialyzed against 250 ml PBS at 25°C for 1 hour to remove the free iohexol. In these experiments, 1 ml liposomal iohexol formulations was placed in a 300,000 MWCO dialysis bag with 4 ml of human plasma, and dialyzed against 250 ml PBS at 37°C (1:4 ratio was chosen). One ml of the external phase was removed at 0, 1, 2, 3, 4, 5, 6 and 8 hours respectively, and analyzed by the UV-vis absorption. Since plasma components also leak from the dialysis bag and have a finite absorbance at 245 nm, a control experiment, where a PBS-plasma mixture is dialyzed against PBS, was also performed. The absorbance of the external phase was subtracted from that for the liposomal iohexol formulation experiments and the resulting absorbance traces can be representative of the leakage of iohexol from

liposomal iohexol formulations. The results showed that the liposomal iohexol formulation was stable in PBS and in human plasma.

[0068] The example leakage curves of iohexol **300** is shown in **Figure 3**. The example liposomal iohexol formulation (30 mg I/ml) was dialyzed against 250 ml of PBS at 4°C. At example time points **305** of 0, 1, 2, 3, 8, 24 hours, and 3, 4, 5, 6, 8, 10 and 18 days, the dialysate was tested for the amount of iohexol. The example leakage curve **310** was obtained by drawing a line through the data at each time point. The data show that the curve stabilized after 1 hour of dialysis. Liposomal iohexol exhibited a leakage of 7.4% of the total encapsulated iohexol over 8 hours, and 7.8% for 18 days by equilibrium dialysis at 4°C. The shelf life of liposomal iohexol formulation therefore can be longer than 18 days.

[0069] The leakage curves of an example iohexol-plasma mixture **400** is shown in **Figure 4**. Liposomal iohexol that had previously been dialyzed against PBS for 1 hour was used in this study to determine the contribution of plasma to leakage of iohexol from the liposomes. At example time points **405** of 0, 1, 2, 3, 8, 24 hours, and 3, 4, 5, 6, 8, 10 and 18 days, the dialysate was tested for the amount of iohexol. The example leakage curve **410** was obtained by drawing a line through the data at each time point. The data show that the curve stabilized after 3 hours, and the liposomal iohexol formulation exhibited a leakage of 2.3% of the total encapsulated iohexol for the 8 hour period, beyond the leakage observed during storage in PBS. Together, these results indicate that the liposomal iohexol formulation can be about 90% encapsulated when stored for 18 days and then injected.

Example 3. *In Vivo* Studies Using Imaging of PEGylated Liposomes Containing Iohexol in a Rabbit

[0070] A female rabbit weighing 2.2 kg was anesthetized with 35 mg/kg ketamine and 5 mg/kg xylazine given intramuscularly, followed by 2% isoflurane vapor given by face cone. After tracheal intubation and placement of venous catheter in an ear vein, 20 mg pentobarbital was given intravenously. The animal's lungs were ventilated using a pressure control ventilator set to peak airway pressure of 15 cm H_2O, and 25 breaths min^{-1}. After transport to the CT scanner, the animal was given 0.25 mg of pancuronium (muscle relaxant) to insure minimal motion during the image acquisition. Supplemental pentobarbital was given every 30-60 minutes, 10-20 mg per dose. An initial volume image of the chest and abdomen was obtained using a 4 slice Phillips MX8000 MDCT scanner in spiral scanning mode, (100 mAs, 120 keV) with a single slice equivalent pitch of 1.25, and a slice

collimation and thickness of 1.3 mm. Images were reconstructed into a 512 x 512 matrix using a standard reconstruction kernel (the "B" kernel). A 0.5 second gantry rotation speed was used. During each imaging protocol, the rabbit was held apneic with airway pressure fixed at 20 cm H_2O (e.g. near total lung capacity) using an underwater bubbler tube on the exhalation port. Next, 15 ml of 34 mg I/ml liposomal iohexol formulation was hand-injected followed by a repeat volume image, then a second injection of 15 ml of liposomal iohexol formulation suspension was followed by a third volume image. A total dose of 475 mg iodine per kg was given in the two injections. Repeat volume images were then initiated at approximately 12, 60, 90, 120, 150 and 180 minutes after the second contrast injection. Following the last image acquisition (~ 3.5 hr post injection of contrast agent), the animal was euthanized with an overdose of pentobarbital and a final, high resolution image was obtained with no motion artifact (with the same airway pressure and image acquisition settings). Finally, an ultrahigh resolution scan was obtained using an ultra sharp reconstruction kernal ("D" kernal and a 1024 x 1024 image matrix) to evaluate anatomic detail without the presence of cardiogenic motion.

Example 4. Image Reconstitution

[0071] Subsequent offline example reconstructions were performed for each of the scans obtained as described in Example 3 with the smallest field of view (5 cm x 5 cm, 0.1 mm voxel size) for 3D viewing of the heart. The enhanced heart chambers were visualized by selecting appropriate settings of the volume rendering software present on the Philips MXV workstation software (version 4.1). Once the settings were established, the same rendering and display settings were used for all time points. Additional structures were segmented at various time points.

[0072] Quantitative analysis was performed by locating regions of interest (ROI) in the aorta, heart, kidney (core and cortex), liver, muscle and spleen. Mean Hounsfield units (HU) were determined at each time point to enable tracking of any decay in contrast concentration with time in each of these structures. Slice and slice location of the ROI's were adjusted for minor variations in anatomic configuration of the rabbit from time point to time point.

Example 5. Time-Attenuation of PEGylated Liposomes Containing Iohexol *In Vivo*

[0073] The example image analysis described in Example 4 was performed at regions of interest in the aorta, kidney (medulla and cortex), liver parenchyma, back muscle, left main coronary artery, pulmonary artery, and in the main stem bronchus (as a control value) and

plotted over time in a graph **500** (**Figure 5**). Mean attenuations (Hounsfield units) were determined at the time points stated in Example 3 to quantify the decay in contrast with time in each of these locations. The data show the enhancement and maintenance of contrast over time in various regions of interest. The average attenuation in the aorta **505**, pulmonary artery **515** and liver cortex 3.5 hours post contrast injection attenuation was 200 HU (enhancement 130 HU), and in the kidney cortex **525** the attenuation was 75 HU (enhancement 25 HU). Attenuation in the blood pool rose rapidly post-injection, and remained virtually constant for the 3.5 hours of study. A slight increase in attenuation in the liver parenchyma **520** was observed. A transient increase in the kidney core **530** was observed, indicating early clearance with little to no clearance later in the study. The small region of interest placed over the left main coronary artery indicated attenuation of 9 HU at base line and peaked at a value of 118 HU. **Figure 6** shows 0 hour baseline **605** and peak enhanced **610** images obtained 2 hours 18 minutes post liposomal injection at the level of the liver. **Figure 6** also shows 0 hour baseline **615** and peak enhanced **620** images obtained 2 hours 18 minutes post liposomal injection at the level of the mid-heart.

[0074] These data indicate the residence time of example PEGylated liposome formulations, which provided contrast enhancement, to be more than 3 hours. Additionally, the data show that contrast enhancement in muscle can be low, indicating the liposomal iohexol can be retained in the blood vessels and does not rapidly extravasate. Additionally, the contrast enhancement in the liver parenchyma indicated that clearance of the composition may substantially be due to the liver, and not the kidneys.

Example 6. *In Vivo* Images of Heart After Administration of PEGylated Liposomes Containing Iohexol

[0075] Additionally, example images of the rabbit heart were analyzed **700** (**Figure 7**), **800** (**Figure 8**), **900** (**Figure 9**) and **1000** (**Figure 10**). **Figure 7** shows volume rendered images of the whole rabbit, before **705** and 2 hours 18 minutes after injection of the liposomal iohexol formulation **710**. Enhancement to the vasculature **715** due to the liposomes can be seen. The results show that, even more than 2 hours after injection, the blood vessels can be visible **715** while, using the same display and rendering parameters, they may not be visible before liposome administration. This enhancement can persist up until the time that the animal is euthanized at more than 3 hours after injection of the second dose of liposomes.

[0076] **Figure 8** shows volume images of the rabbit heart acquired pre-contrast **805** and at 20 minutes **810**, 1 hour 15 minutes **815**, 1 hour 51 minutes **820**, 2 hour 38 minutes **825**, and 3 hour 23 minutes **830** after administration of the liposomal iohexol formulation. All display and rendering parameters are identical for all images. The anatomies of all four heart chambers can be distinctly visualized along with the associated great vessels. Note that there may be absence of blood pool in the upper left panel **805** and the persistent enhanced opacity of the blood pool up to the final panel representing 3 hours 23 minutes post injection **830**. Visible structures include: right ventricle **835** (RV); left ventricle **840** (LV); Aorta **845** (Ao); pulmonary artery **850** (PA); and the inferior vena cava **855** (IVC). These images demonstrated sustained contrast even 3 hours after administration of the liposomal iohexol.

[0077] **Figure 9** shows a thick-slab rendering of the heart obtained at ultrahigh resolution after the rabbit was euthanized and thus cardiac motion was eliminated. Labeled structures include the right ventricle **905** (RV); left ventricle **910** (LV); and aorta **915** (Ao).

[0078] **Figure 10** shows images of the left coronary artery of a rabbit under high magnification conditions at 3 hours after the second injection of the liposomal iohexol formulation. The left panel **1005** shows a 1.3 mm thick CT slice of *in vivo* rabbit heart imaged 3 hours 18 minutes after the second injection of one embodiment of liposomal iohexol. The right panel **1010** shows a volume rendered view of the same data set. The left coronary artery (shown as **1015** in **1010**) was enhanced by 109 HU.

[0079] The above descriptions have referred to the preferred embodiments and selected alternate embodiments. Modifications and alterations will become apparent to persons skilled in the art upon reading and understanding the preceding detailed description. It is intended that the embodiments described herein be construed as including all such alterations and modifications insofar as they come within the scope of the appended claims or the equivalence thereof.

TAB C

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Poly(ethylene glycol)-coated anti-cardiac myosin immunoliposomes: factors influencing targeted accumulation in the infarcted myocardium

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Abstract

Biodistribution and infarct accumulation of different liposome preparations in rabbits with experimental myocardial infarction have been investigated. The influence of such parameters as liposome size, and presence or absence of poly(ethylene glycol) (PEG) and infarct-specific antimyosin antibody (AM) on liposome behavior in vivo was studied. All three variables were shown to affect liposome biodistribution, liposome size being the least significant variable. Statistical analysis of the data obtained demonstrated that of all variables, PEG coating expresses the strongest influence on the liposome blood clearance, significantly ($P = 0.0001$) increasing the mean level of blood radioactivity under all circumstances. Infarct accumulation depended upon the presence of both PEG ($P = 0.0013$) and AM ($P = 0.005$). The infarct-to-normal ratio was affected by the presence of AM ($P = 0.0002$), but the extent of the effect depended also on the presence of PEG ($P = 0.01$). Two differing mechanisms can be seen in infarct accumulation of PEG-liposomes (slow accumulation via the impaired filtration) and AM-liposomes (specific binding of immunoliposomes with the exposed antigen). Both mechanisms are supplementary in case of liposomes carrying PEG and AM at the same time. An optimization strategy is suggested for using liposomes as carriers for diagnostic (a high target-to-nontarget ratio is required) and therapeutic (a high absolute accumulation in the target is required) agents

Keywords: Liposome; Immunoliposome; Long-circulating liposome; Antimyosin; Antibody; Myocardial infarction; Drug targeting

1. Introduction

The targeting of pharmaceuticals to the heart is aimed at two main objectives: diagnostic imaging and the delivery of therapeutics to the damaged myocardium. Liposomes have been shown to serve as convenient carriers for both diagnostic and therapeutic pharmaceuticals. Spontaneous accumulation of positively charged liposomes in the regions of experimental myocardial infarction was described by Caride in 1977 [1]. Further experiments [2–4] demonstrated that liposome accumulation in ischemic tissues is a general phenomenon and might be explained by impaired filtration in these areas, resulting in trapping of liposomes within the ischemic zones [5]. This observation led to the conclusion that drug-loaded liposomes can be used for 'passive' drug delivery into the ischemic tissues, particularly into the infarcted myocardium [6,7]. Thus, liposomes loaded with a thrombolytic enzyme, streptokinase, were able to accelerate thrombolysis and reperfusion in a canine model of myocardial infarction [8]. Liposomes with superoxide dismutase were reported to be more effective in preventing ischemic and reperfusion injuries in different tissues, including myocardium, compared to the native enzyme [9,10]. Furthermore, it was shown that liposomes loaded with sodium or calcium ions have demonstrated significant influence on the electrical activity of cultured heart cells from chicken embryos [11]. Similarly, liposomes with entrapped ATP were shown to normalize ischemic conditions in some tissues [12].

To improve the efficiency of liposomal drug delivery

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into the ischemic myocardium utilisation of antibody-mediated liposome targeting was proposed. Antimyosin antibody [13,14] has been used for 'active' targeting of liposomes to infarcted myocardium [15]. This targeting phenomenon is based on the observation that normal myocardial cells with intact membranes do not permit extracellular macromolecules, such as antimyosin antibody, to traverse the cell membrane. However, necrotic cardiomyocytes with disrupted membranes can no longer prevent the antibody from interacting with myosin [16]. This forms a basis for targeted delivery of pharmaceuticals into the affected myocardium [17]. Antibody to canine cardiac myosin covalently coupled to liposomes has been successfully used for targeting of the experimental myocardial infarction in dogs [15]. Incorporation of radioactivity within liposomes also allowed for visualization of the necrotic myocardium [15].

However, 'passive' liposome targeting or 'active' targeting with immunoliposomes can not provide high enough accumulation of the targeting agent in the areas with limited blood supply (such as an infarct zone) due to fast sequestering of liposomes by the reticuloendothelial system (RES), resulting in insufficient contact time with the target. Some in vivo properties of liposomes, such as circulation time, can be improved by grafting their surface with certain flexible polymers [18–20]. One of the most promising approaches for increasing liposome circulation time is coating them with poly(ethylene glycol), or PEG. PEG decreases the rate of opsonization of liposomes and therefore their recognition by liver cells, which significantly increases liposome circulation time in the blood [21,22]. Moreover, long-circulating polymer-coated liposomes can be made target-specific' by co-incorporation of a specific antibody on the liposome surface, as described earlier [23]. In that study, antimyosin-modified PEG-liposomes had the abilities to recognize and bind the target, as well as circulate long enough to provide high target accumulation. The next step in this endeavor is to optimize liposome properties for the delivery of diagnostic and therapeutic agents into the damaged myocardium. For diagnostic purposes maximum target-to-nontarget ratio is essential; whereas, for therapeutic purposes the maximum absolute dose delivery of the liposomal contents into the affected tissue is more important. However, with highly toxic pharmaceuticals, high background is undesirable and low non-specific accumulation could be required. With these aims in mind, in vivo properties and biodistribution of PEG and/or antimyosin antibody-coated liposomes of different sizes were investigated in rabbits with experimental myocardial infarction.

We report here that in the rabbit myocardial infarct model, the size of the liposomes has limited influence on their in vivo behavior and accumulation at the target organ. The presence of antibody and/or PEG on the liposome surface is more critical resulting in differences which may influence whether its use should be in diagnosis or therapy.

2. Materials and methods

2.1. Materials

Diethylenetriaminepentaacetic acid (DTPA) cyclic anhydride, monomethoxy poly(ethylene glycol) succinimidyl succinate (PEG-OSu), M_r approx. 5000, 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDC), octyl glucoside (OG), and cholesterol were purchased from Sigma Co. (St. Louis, MO). Dioleoylphosphatidylethanolamine (PE), egg yolk phosphatidylcholine (PC), and *N*-glutarylphosphatidylethanolamine (NGPE) were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-Hydroxysulfosuccinimide (HSSI) was from Pierce (Rockford, IL). Carrier-free ^{111}In as InCl3 was obtained from Amersham (Arlington Heights, IL). Mouse monoclonal antibody R11D10 or 2G4-2D7 specific for cardiac myosin heavy chains was prepared as described [16].

2.2. Methods

Synthesis of PEG-PE. The synthesis of PEG-PE was performed as described in [21]. Briefly, an aliquot of PEG-OSu was added to a solution of PE in chloroform, followed by addition of triethylamine (PEG-OSu/PE/triethylamine = 3:1:3.5, mol/mol). The reaction mixture is incubated overnight at room temperature and the chloroform is evaporated with a stream of nitrogen gas. The reaction mixture is than redissolved in 0.145 M NaCl. Unreacted PEG-OSu is rapidly hydrolyzed in the aqueous media. The resulting mixture in saline is applied to a Bio-Gel A1 5m column equilibrated with saline. Peak fractions containing PEG-PE micelles eluted in the void volume are pooled, dialyzed against water and lyophilized.

Preparation of antibodies and their fragments. Monoclonal antibodies were purified according to the standard method from corresponding murine ascites by ammonium sulfate precipitation, DEAE-cellulose anion exchange chromatography, and chromatofocusing over a pH gradient of 7.0 to 5.0 [16]. Antibody preparations were characterized by gel electrophoresis and HPLC. Fab fragments were used instead of whole antibodies in order to decrease Fc-mediated uptake of liposome-whole antibody conjugates by cells of the reticuloendothelial system (RES). The digestion of IgG and Fab purification was performed as follows: (a) 0.5 ml of the 50% slurry of immobilized papain (Pierce) were washed two times with 4 ml of digestion buffer (42 mg of cysteine/12 ml of phosphate buffer (pH 7.0)) for equilibration, and then suspended in 0.5 ml of the same buffer; (b) 0.5 ml of IgG sample (10–20 mg protein/ml) were diluted with 0.5 ml of the digestion buffer and added to the tube with immobilized papain suspension; (c) incubation proceeded for overnight depending at 37°C with intensive stirring; digestion degree was monitored with HPLC; (d) digested Fab and Fc fragments, and non-digested IgG were separated from papain

gel; (e) column with 5 ml of protein A-Sepharose (Pharmacia) was equlibrated with phosphate-buffered saline (PBS) (pH 8.0), and digested IgG sample was applied; (f) column was washed with PBS, Fab fragment was collected (purity was checked by HPLC); (g) Fc fragment was washed away with 0.1 M glycine (pH 3.0) to regenerate the column.

Antibody modification. For the incorporation into the liposomal membrane, antimyosin antibody Fab was initially modified with the hydrophobic anchor, NGPE, as described in [24]. Briefly, 0.3 mg of NGPE were dried with argon from chloroform solution and then solubilized with 0.5 ml of 0.016 M OG in 50 mM Mes. The solution was supplemented with 12 mg EDC and 15 mg of HSSI. The mixture was incubated for 5 min and then added to the solution of 2 mg of Fab in 0.1 M Hepes (pH 7.6). pH was adjusted to 8.0 with 1 M NaOH, and the mixture was incubated overnight at 4°C. The modified antibody was purified by dialysis against HBS.

Preparation of liposomes. Liposomes were prepared by the detergent (OG) dialysis from the mixture of PC and cholesterol (7:3 molar ratio). 6 mol% of PEG-PE and 1 mol% of DTPA-SA were added to the lipid mixture, which was then argon-dried, vacuumed, solubilized with OG in Hepes-buffered saline (HBS) (pH 7.4) (final total lipid concentration may vary from 5 to 20 mg/ml), and dialyzed overnight against HBS at 4°C. When necessary, 0.01 mol% of NGPE-antimyosin Fab was added to OG-solubilized lipid mixture to prepare targeted liposomes. Modification with NGPE normally allows the binding of several hundred protein molecules per single 250 nm liposome [24]. As was shown with ^{125}I-labeled antibody, in our particular case the efficacy of protein binding varied between 65 and 75% and did not depend on the presence of PEG-PE in the lipid mixture. The unbound antibody was separated on a Bio-Gel A15m column as in [23]. Liposomes obtained were sized by passing through the polycarbonate filters with pore size of 0.6, 0.4, and 0.2 μm (Nuclepore). Liposomes of two sizes were prepared: 120–150 nm (small liposomes, SL) and 350–400 nm (large liposomes, LL). Actual size and size distribution of liposomes were determined with Coulter N4 MD Submicron Particle Size Analyzer (Coulter Electronics).

For biodistribution studies, liposomes were radioactively labeled with ^{111}In via liposome-incorporated amphiphilic chelating agent diethylenetriaminepentaacetic acid-stearylamine (DTPA-SA). DTPA-SA was synthesized according to recommendations of [25] with some changes. Briefly, 1.55 g of SA and 2.5 g of DTPA cyclic anhydride were mixed with 250 ml of dry chloroform. The mixture was refluxed for 1 h, the top outlet of the system being coated with foil. Then, 3 ml of triethylamine were added, and the mixture was refluxed for an additional 48 h. Chloroform was evaporated on the rotor evaporator, it's traces were removed by incubating the flask in the water bath at 50°C. 100 ml of 0.1 M HCl were added to the dry product, and the mixture was stirred with heating at 80°C for 10 min and stored overnight at room temperature. The precipitate was separated by centrifugation, washed three times with 100 ml of 0.1 M HCl with stirring, and then lyophilized. The lyophilized product was washed twice with 100 ml of methanol, and then recrystallized twice from boiling methanol and dried.

DTPA-SA loading with ^{111}In was performed after its incorporation into liposomes via the transchelation mechanism. For this purpose, the liposome suspension (normally, 2 ml) was supplemented with 30 μl of 1.0 M citrate and incubated for 1 h with required quantity of citrate complex of ^{111}In at room temperature, and then dialyzed overnight against HBS at 4°C to remove free label.

Determination of antibody-liposome immunoreactivity by direct binding of radiolabeled antibody-liposome and antibody-liposome-polymer conjugates. Microtiter plates were coated with 50 μl of 10–50 μg/ml of dog cardiac myosin and incubated at 4°C for 12–18 h. The antigen solution was removed and the wells were filled with 1% horse serum in 0.15 M phosphate-buffered saline (pH 7.4) to saturate the remaining non-specific binding surfaces of the microtiter wells. The solution was removed after a 4 h incubation at room temperature and the wells were washed extensively with standard washing solution. To the antigen-coated wells prepared as described above, serial dilutions of ^{111}In-labeled antibody-polymer-liposome preparation were added. The maximum count used per 50 μl aliquot was $2 \cdot 10^5$ cpm. Half dilutions were made until the aliquots contained approximately 10000 cpm per 50 μl. The reaction was allowed to proceed until equilibrium, for 4 h at room temperature or overnight at 4°C. The wells were extensively washed to remove slightly-bound radioactivity, cut and counted in a γ-scintillation counter for ^{111}In activity. Binding of ^{111}In-labeled antibody-polymer-liposome conjugates was compared with the binding of corresponding antibody directly labeled with ^{111}In via DTPA technique.

Experimental myocardial infarction in rabbits. Rabbits (New Zealand White rabbits, 2–3 kg) were anesthetized with Ketamin (70–75 mg/kg) and Xylazine (7–7.5 mg/kg). Right femoral artery cut-down was performed to establish a blood pressure line and for arterial blood sampling. The right femoral vein was catheterized to allow intravenous medication. A tracheostomy was performed, and ventilation was instituted through an endotracheal tube with a Harvard Rodent Ventilator (model 683). After artificial respiration, the anesthesia was switched to 3 ml (19.5 mg) pentobarbital infusion per hour. A left thoracotomy was performed and the anterior descending coronary artery was occluded with a silk suture placed through the myocardium with an SH-needle. After 40 min, the snare was released to allow reperfusion. A radiolabeled liposome preparation (2–3 ml in HBS, up to 30 mg of total lipids and 200 to 500 μCi of ^{111}In) was injected intravenously within 30 min of reperfusion. Blood samples were taken

Table 1
Biodistribution of liposomes (5 h post-injection, % dose/g)

Size	AM	PEG	Blood		Liver		Spleen		Kidneys		Lungs		Infarct		Normal		Infarct-to-normal	
			mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.
S	–	–	0.06	0.01	0.80	0.15	0.85	0.68	0.01	0.002	0.02	0.003	0.02	0.01	0.004	0.001	5.17	2.35
S	+	–	0.16	0.10	0.37	0.16	0.55	0.15	0.02	0.01	0.03	0.01	0.14	0.05	0.007	0.002	22.70	2.38
S	–	+	0.50	0.11	0.16	0.06	0.42	0.11	0.03	0.01	0.13	0.06	0.13	0.10	0.017	0.006	8.05	5.03
S	+	+	0.35	0.11	0.13	0.03	0.26	0.14	0.05	0.04	0.15	0.12	0.25	0.14	0.02	0.01	14.10	7.15
L	–	–	0.06	0.03	0.70	0.25	0.92	0.73	0.01	0.01	0.07	0.03	0.02	0.01	0.01	0.01	4.03	1.27
L	+	–	0.10	0.05	0.39	0.09	0.47	0.28	0.03	0.01	0.06	0.04	0.09	0.04	0.003	0.001	29.67	23.76
L	–	+	0.38	0.02	0.30	0.14	0.47	0.09	0.05	0.03	0.11	0.03	0.14	0.04	0.02	0.01	7.38	1.67
L	+	+	0.41	0.08	0.47	0.38	0.60	0.32	0.04	0.02	0.16	0.04	0.15	0.02	0.02	0.004	10.43	3.30

The mean infarct-to-normal ratios were calculated from the ratios of the original values. They need not to be equal to the ratios of the means given in the table. All values were rounded off to 2 or 3 decimal places. See Section 2 for details.

serially at 1, 3, 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min after injection of liposomes to measure blood radioactivity (liposomal clearance). Time within which the blood content of the liposome-associated radioactivity dropped to 50% of the injected dose was designated as $t_{1/2}$. 5 h after liposome injection, animals were killed by an overdose of pentobarbital. The heart was excised and cut into 5 mm slices, stained with 2% triphenyl tetrazolium chloride to identify necrotic areas. Each slice was further divided into smaller segments for biodistribution studies following a standard scheme [23]. Samples of normal and infarcted myocardium, and other organs of interest were dried from excessive blood by blotting on absorbant towels and then weighed and counted in a gamma-counter. The liposome accumulation in the heart was expressed as infarct-to-normal myocardium radioactivity ratio. Biodistribution of liposomes was also studied following the liposome-associated radioactivity accumulation in nontarget organs (such as liver, spleen, kidneys and lung), and expressed as %dose per g of the tissue.

Statistical treatment of the animal data. The liposomes were prepared varying three factors: size (SL and LL), PEG coating and antimyosin antibody (AM) coating. Eight subgroups of animals were studied, four for each SL and LL groups. The four subgroups for each size included plain liposomes, PEG(only)-coated liposomes, AM(only)-coated liposomes, and PEG-AM(coincorporated) liposomes. Each subgroup comprised 3–5 rabbits. The contribution of each of the three experimental factors to the infarct-to-normal myocardium radioactivity ratio and the liposome accumulation in the liver, spleen, kidney, and lung were analyzed with a three-way factorial analysis of variance. Each model was reduced to the significant terms ($P < 0.05$), and all effects were estimated. They are presented as estimate (± standard error). Means of the observed values were used to present the biodistribution data, while means of the corresponding ratios were used to estimate infarct accumulation (Table 1).

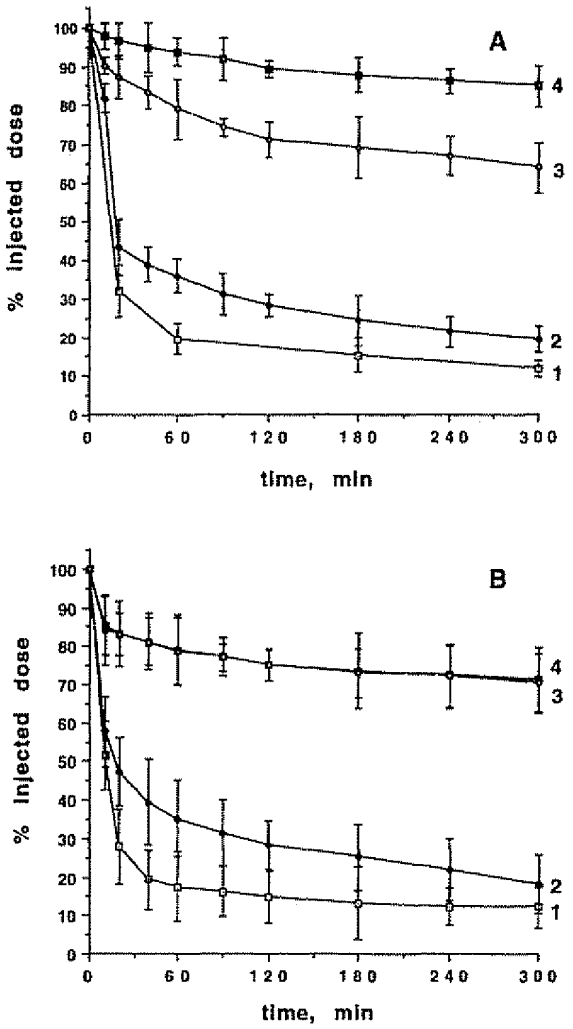

Fig. 1. Blood clearance of different liposomes in rabbits with experimental myocardial infarction. (A) Small liposomes. 1, SL; 2. AM-SL; 3. PEG-AM-SL; 4. PEG-SL. (B) Large liposomes. 1. LL; 2. AM-LL; 3. PEG-AM-LL; 4. PEG-LL.

3. Results

3.1. Immunoreactivity

The modification of antimyosin R11D10 Fab with hydrophobic substituent NGPE, and incorporation of NGPE-Fab into the liposomal membrane in this particular case decreased antibody immunoreactivity by approximately 15- to 20-times. The appropriate binding constant decreased from $5 \cdot 10^8$ for the native Fab to $3 \cdot 10^7$ for NGPE-Fab, and to $2 \cdot 10^7$ M^{-1} for an individual NGPE-Fab in liposome. However, as demonstrated earlier, such a decrease in immunoreactivity of an individual antibody is at least partially compensated by the increase in avidity due to the presence of multiple antibody molecules on the liposome surface providing specific multipoint attachment of immunoliposome to the target [26]. As a result, the apparent constant of immunoliposome binding to the antigen was in the range $(3–6) \cdot 10^8$ M^{-1} liposomes.

3.2. Blood clearance

The patterns of clearance of different liposome preparations from the circulation in infarcted rabbits are presented in Fig. 1.

3.3. Biodistribution

The biodistribution data obtained after 5 h of experiment were expressed as % of injected dose per gram of

tissue. The statistical analysis of the data related to individual organs revealed the following (see also Table 1):

Blood. The blood radioactivity at 5 h depended significantly upon the interaction (combination) of all three factors ($P = 0.006$). The strongest effect was observed for the PEG coating, which significantly ($P = 0.0001$) increased the mean level under all circumstances. The estimated effect was 0.44 (± 0.06) for SL without AM coating, 0.19 (± 0.06) for AM-SL, 0.32 (± 0.06) for LL without AM, and 0.31 (± 0.06) for AM-LL.

Infarcted area. In the infarcted myocardium, accumulation depended significantly upon the presence or absence of both the PEG coating ($P = 0.0013$) and the antimyosin antibody ($P = 0.005$). Both coatings increased the mean infarct accumulation: PEG - 0.10 (± 0.03), and AM - 0.09 (± 0.03); for all PEG-containing vs. corresponding PEG-free, and AM-containing vs. corresponding AM-free samples, respectively. Minimum accumulation was observed for both SL and LL - 0.02 ± 0.01; maximum accumulation was found for PEG-AM-SL - 0.25 ± 0.14.

Normal myocardium. Normal myocardium was demonstrably affected only by the PEG coating ($P = 0.0001$). The coating was estimated to increase the mean value by 0.013 (± 0.002).

Infarct-to-normal ratio. The infarct-to-normal ratio was demonstrably affected by the presence or absence of antimyosin antibody on the liposome ($P = 0.0002$). However, the extent of the effect depended significantly upon the presence or absence of the PEG coating together with AM ($P = 0.01$). In liposomes without any PEG coating, the AM coating increased the mean ratio by 21 (± 4). In liposomes with the PEG coating, the AM coating is estimated to increase the mean ratio by only 5 (± 4). This effect is not statistically significant ($P = 0.22$). Maximum value of the infarct-to-normal ratio was achieved for AM-SL - 22.70 ± 2.38. The value for AM-LL (29.67) can not be considered as reproducible because of large standard deviation (23.76).

Liver. Liver radioactivity accumulation was affected by both the PEG and AM coatings. Liposomes with PEG coating alone, the AM coating alone, or both had significantly ($P = 0.007$, 0.0003, and 0.0005, respectively) lower mean liver activity values than liposomes with neither coating. The differences can also be noticed between the mean values of accumulation of liposomes with either or both coatings (see Table 1).

Spleen. Spleen accumulation was not demonstrably affected by any of the three factors (size, PEG, AM).

Kidney. Radioactivity accumulation in the kidney was shown to be affected only by the PEG coating ($P = 0.002$) which was estimated to increase the mean value by 0.028 (± 0.008).

Lung. Radioactivity accumulation in the lung was shown to be affected only by the PEG coating ($P = 0.0003$) which was estimated to increase the mean value by 0.09 (± 0.02).

4. Discussion

Numerous in vitro and in vivo studies already have dealt with the interaction of long-circulating liposomes with cells and organs [27–29]. These studies have clearly documented the dependence of liposome clearance rate and the biodistribution pattern on liposome size and the presence of the protective substances on the liposome surface. However, attempts to prepare long-circulating *immuno*liposomes have added one more variable to the system, the targeting moiety (such as a monoclonal antibody). At a given phospholipid composition, the properties of the long-circulating immunoliposomes would depend on the liposome size, and the presence of both, the protective substance and antibody on the liposome. Therefore, permutations of these variables should enable the control of liposomal characteristics, such as clearance time, biodistribution, and target accumulation. This should permit preparation of liposomes specifically designed for targeted delivery of diagnostic agents (where maximum liposome accumulation in the target area and minimum accumulation in normal tissues are required to provide maximum target-to-nontarget ratio) and of therapeutic agents (where maximum absolute accumulation of drug-loaded liposomes in the target is required).

4.1. Blood clearance characteristics

Small liposomes. In case of SL, plain liposomes demonstrate the fastest clearance. Addition of AM slightly increases the circulation time probably by reducing the unaltered surface of liposomes which determines the liposome accessibility to opsonons [19,30]. Since we used Fab fragment of the antibody, immunoliposome capture via Fc fragments did not occur as in case of the whole IgG. Grafting of PEG to the liposome surface, as expected [21,23], sharply increases liposomal circulation time. Simultaneous incorporation of AM and PEG also significantly increases the circulation time, the blood clearance was marginally faster compared to the PEG liposomes. This difference in the clearance can be partly explained by a more pronounced interaction between AM and plasma proteins than between PEG and the same proteins. However, significantly longer circulation of PEG-AM-SL as compared to AM-SL allows for effective targeting as described by us previously [23].

Large liposomes. The clearance characteristics of LL are similar to SL. The only exclusion is that AM Fab incorporation practically does not influence circulation time of PEG-LL. This can be explained by size difference between SL and LL; the surface area of LL is approximately 6 times larger than that of SL, and possible irregularities in PEG location (such as the hypothetical formation of more dense PEG clusters because of polymer-to-polymer interactions which can result in the appearance of polymer-free areas on the liposome surface exposed for the

Table 2
Liposome circulation time

Liposome	Variable	$t_{1/2}$ (min)	Comments
Small	plain	10–15	Very short $t_{1/2}$
	+ AM (Fab)	15–20	Slight increase (due to partial surface protection?)
	+ PEG	> 1000	Significant increase (full surface protection)
	+ AM / + PEG	ca. 600	Intermediate increase (interaction with plasma proteins is stronger for Fab than for PEG)
Large	plain	ca. 10	Very short $t_{1/2}$
	+ AM (Fab)	ca. 15	Slight increase; same as for SL
	+ PEG	> 600	Significant increase in circulation time, but still less than for PEG-SL. No difference between PEG- and PEG-AM-LL; possible irregularities in surface PEG distribution (and consecutive opsonization) are more probable for LL
	+ AM / + PEG		

opsonization) are more probable for PEG-LL than for PEG-SL. This is why the circulation time for PEG-LL is less than that for PEG-SL, and additional incorporation of AM Fab onto the surface of LL does not significantly change the clearance. Some alternative explanations are, probably, also possible.

The circulation time is therefore strongly influenced by all three factors studied – liposome size and the presence of AM, PEG or both on the liposome surface (see Table 2). However, size difference seems to be less important than surface modification with PEG or AM.

4.2. Targeting of the infarcted myocardium

Small liposomes. There is a marginally higher localization of plain SL in the infarcted myocardium as compared with remote normal myocardium. Such non-specific localization of liposomes in the injured myocardium has been shown previously [1–5]. Interestingly, both PEG-SL and AM-SL accumulate in the necrotic area almost identically if the total is expressed in absolute quantities, 0.13 and 0.14% dose/g, respectively. This indicates two different mechanisms for liposomal accumulation. Firstly, the specific one, which requires the presence of antibodies on the surface of short-circulating liposomes and permits selectively intense targeting of necrotic myocardium even after few passages over the area of interest [15]. Second mechanism is likely to be a non-specific, which slowly proceeds via impaired filtration mechanism in affected tissues with leaky vascular endothelium and requires repeated passages of liposomes through the target, i.e., prolonged circulation. The phenomenon of the accumulation of long-circulating liposomes has been reported previously in tumors with highly permeable endothelial layer [31,32], where the necrotic zone (if any) may also suffer from impaired drainage still further facilitating liposome accumulation.

Although absolute accumulation of AM-SL and PEG-SL is similar, the infarct-to-normal ratio (or relative targeting) is much higher for AM-SL than for PEG-SL; 22.70 ± 2.38 versus 8.05 ± 5.03, respectively. The reason for this interesting phenomenon is that the non-specific accumulation of AM-SL in normal tissues is very low (the time of AM-SL residence in the blood is too short). On the other hand, long-circulating PEG-SL slowly and non-specifically accumulate in both infarcted and normal tissues but the accumulation in infarcted tissued is comparatively higher due to exaggerated vascular permeability.

The combination of AM and PEG (PEG-AM-SL) on the liposome surface adversely affects the target-to-normal ratio, which is lower than that of AM-SL due to higher non-specific capture of PEG-AM-SL in normal tissue. However, in absolute terms (% dose/g) this combination provides excellent accumulation (0.25 ± 0.14) which is 2-fold higher than for short-circulating AM-SL. The intense PEG-AM-SL accumulation in the infarcted tissue can be explained by both specific and non-specific mechanisms of accumulation acting synergistically.

Large liposomes. Although, PEG and AM predominantly affected the target accumulation of liposomes, the liposome size also influences the targeting to some extent. The increase in liposome size reduces their ability for non-specific accumulation in the necrotic tissues. It can simultaneously affect the efficacy of AM-LL interaction with the target; for example, in the case when due to a short contact time with the target, part of fast-clearing AM-LL fail to form a sufficient number of Fab-antigen bonds to firmly anchor a large liposome to the target.

Similar to plain SL, no noticeable accumulation of plain LL occurs in the infarction due to brief residence time. The accumulation of both AM-LL and PEG-LL is higher. In case of LL, absolute accumulation of PEG-LL is even slightly better than that of AM-LL (0.14 ± 0.04 and 0.09 ± 0.04% dose/g, respectively). Evidently, for LL prolonged circulation can yield greater absolute accumulation through gradual accumulation than short-term specific interaction, part of which can be non-productive because of the large liposome size. Comparing these data with that of SL, one can hypothesize that to overcome the disadvantage of liposome size, at least a minimum critical residence time may be required for immunoliposome targeting.

Table 3
Liposome targeting to infarcted myocardium

Liposome	Variable	% dose/g	Comments
Small	plain	0.02	Very low accumulation
	+AM (Fab)	0.14	Good and similar. *Two mechanisms work: (1) specific binding and (2) non-specific accumulation due to impaired filtration.* Relative targeting (target-to-normal ratio) is much better for AM-SL (22.7) than for PEG-SL (8.05), because of low accumulation of AM-SL in normal tissues (fast clearance) and high accumulation of PEG-SL (slow clearance)
	+PEG	0.13	
	+AM; +PEG	0.25	*Absolute accumulation is maximal; 0.25% dose / g; both mechanisms work!* Relative targeting is intermediate (14) because of high non-specific accumulation in normal tissues
Large	plain	0.02	Very low accumulation
	+AM (Fab)	0.09	Good and relatively close accumulation. Prolonged circulation might be even more effective than short-term specific interaction. Relative accumulation for AM-LL is again higher than for PEG-LL because of non-specific accumulation of PEG-LL in normal tissue
	+PEG	0.14	
	+Ab; +PEG	0.15	No improvement compared to PEG-LL: limiting step is non-specific accumulation in normal tissues; no room for AM additive effect.

In spite of relatively low AM-LL accumulation infarct-to-normal ratio remains higher for AM-LL than for PEG-LL due to essentially no non-specific uptake of AM-LL in normal myocardium (infarct-to-normal ratios, AM-LL 29.67 ± 23.76 vs PEG-LL 7.38 ± 1.67).

Co-incorporation of AM and PEG into the same liposome does not improve absolute accumulation of large liposomes. This can be explained by predominant effect of non-specific accumulation via impaired filtration mechanism which may offset the advantage of specific targeting in the case of LL. Infarct-to-normal ratio for PEG-AM-LL is between that for AM-LL and PEG-LL. Maximum accumulation of PEG-AM-LL is also less than that of PEG-AM-SL.

The maximum infarct-to-normal ratios could therefore be achieved for AM-SL, which makes them attractive for the targeted delivery of the agents where the maximum difference between the area of interest and normal tissues is desired (such as imaging agents). At the same time, for the requirement of maximum absolute delivery within the target tissue, liposomes combining on the surface both a protective polymer and an antibody appear to be the carriers of choice (PEG-AM-SL in our particular case) (Table 3).

4.3. Liposome biodistribution in nontarget organs

The biodistribution of both SL and LL and their surface-modified derivatives reconfirms previously known characteristics (Table 1).

Small liposomes. Plain SL and AM-SL are sequestered predominantly in the liver and spleen followed by a modest capture in kidneys and lungs. PEG modification of liposomes with or without AM modification, significantly decreases liposome accumulation in reticuloendothelial organs, while noticeably increasing it in kidneys and lungs. Pulmonary accumulation can be increased 8-fold.

Large liposomes. The common features of biodistribution pattern are preserved for different types of LL. Similar to the biodistribution of SL, sequestration of plain LL and AM-LL is observed in the liver and spleen. The protective effect of PEG modification is less pronounced than for small liposomes. LL with both AM and PEG on the surface accumulate in the spleen twice as much as SL counterpart. Lung accumulation is 3.5-times higher for plain LL than for plain SL, and increases further for PEG-coated LL. Nonspecific LL distribution in renal tissue was same as that for SL.

From the statistical analysis of the experimental data in the present study some important conclusions can be drawn with reference to the mechanisms of liposome accumulation in the target tissues, and optimization of liposome properties is suggested for their use as vehicles for various pharmaceuticals tailored to clinical demands.

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TAB D

United States Patent [19]

Payne et al.

[11] **Patent Number: 4,744,989**

[45] **Date of Patent: May 17, 1988**

[54] **METHOD OF PREPARING LIPOSOMES AND PRODUCTS PRODUCED THEREBY**

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[51] **Int. Cl.**[4] **A61K 9/16; A61K 37/22; A61K 9/50**

[52] **U.S. Cl.** **424/490; 424/4; 424/5; 424/450; 424/491; 424/493; 514/3; 514/31**

[58] **Field of Search** **514/3, 31; 424/490, 424/491, 493, 450, 4, 5**

[56] **References Cited**

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[57] **ABSTRACT**

A method is provided for preparing stable liposome precursors in the form of a water-soluble carrier material coated with a predetermined amount of a thin film of liposome components, which method includes the steps of dissolving at least one liposome-forming amphipathic lipid, optionally, at least one biologically active compound, and, optionally, at least one adjuvant in a suitable organic solvent and employing the resulting organic solution to coat a suitable water-soluble carrier material to form thin film of predetermined amount of liposome components thereon. Upon exposing the coated carrier material to water, the thin films of liposome components hydrate and the carrier material dissolves to give liposome preparations.

28 Claims, No Drawings

METHOD OF PREPARING LIPOSOMES AND PRODUCTS PRODUCED THEREBY

REFERENCE TO THE APPLICATION

This application is a continuation-in-part of U.S. patent applications Ser. Nos 578,156 filed Feb. 8, 1984 and 578,159, filed Feb. 8, 1984.

FIELD OF THE INVENTION

The present invention relates to a method for preparing particulate water-soluble carrier materials coated with thin films of liposome components (also referred to as proliposomes), which coated carrier materials are employed to form liposome preparations, and to intermediates and products produced in such method.

BACKGROUND OF THE INVENTION

Liposomes are widely described in the literature and their structure is well known. They are formed by amphipathic molecules such as the class II polar lipids, that is, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebrosides. Liposomes are formed when phospholipids or other suitable amphipathic molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material. Another type of liposome is known which is formed of a single bilayer encapsulating aqueous material which may also be referred to as a unilamellar vesicle. "If water-soluble materials are included in the aqueous phase during the swelling of the lipids they become entrapped between the lipid bilayers. Alternatively, lipid soluble materials may be dissolved in the lipid and, hence, may be incorporated into the lipid bilayers themselves," Ryman, B. E., "The Use of Liposomes as Carriers of Drugs and Other Cell-Modifying Molecules," Proc. 6th Int'l. Congr. Pharmacol. 5, 91 (1976), published in "Drug Applications," *Clinical Pharmacology*, Vol. 5, pp. 91–103, Pergamon Press (1975).

In recent years there has been much interest in the use of liposomes as carriers of compounds which are of interest because of one or other biological property, for example, medicaments, proteins, enzymes, hormones and diagnostic agents, hereinafter referred to as "biologically active compounds." Liposomes have been suggested as carriers for drugs, see Ryman, supra at page 91 and Gregoriadis, G., "Enzyme or Drug Entrapment in Liposomes: Possible Biomedical Application," *Insolubilized Enzymes*, Ed. M. Salmona et al, Raven Press, N.T. 1974, pp. 165–177.

Water-soluble materials are encapsulated in the aqueous spaces between the biomolecular layers. Lipid soluble materials are incorporated into the lipid layers although polar head groups may protrude from the layer into the aqueous space. The encapsulation of these compounds can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation of an organic solvent. When this film is dispersed in a suitable aqueous medium, multilamellar liposomes are formed (also referred to as coarse liposomes). Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble biologically active compounds are usually incorporated by dispersing the cast film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysation or some other suitable procedure. Lipid-soluble biologically active compounds are usually incorporated by dissolving them in the organic solvent with the phospholipid prior to casting the film. Providing the solubility of these compounds in the lipid phase is not exceeded or the amount present is not in excess of that which can be bound to the lipid, liposomes prepared by the above method usually contain most of the compound bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required. Other methods of preparing liposomes have been described although these are mainly specialized methods producing unilamellar liposomes and include reverse-phase evaporation of an organic solvent from a water-in-oil emulsion of phospholipid, infusion of organic solutions of phospholipid into large volumes of aqueous phase and detergent removal from mixed micelles of detergent and lipid.

Aqueous liposome dispersions only have limited physical stability. The liposomes can aggregate and precipitate as sediment. Although this sediment may be redispersed, the size distribution may be different from that of the original dispersion. This may be overcome to some extent by incorporation of charged lipids into the liposomes. In addition, on storage the biologically active compounds may be lost into the external aqueous phase which restricts the potential of these preparations as practical dosage forms. This is particularly notable for low molecular weight water-soluble compounds but lipid soluble compounds too can partition into the external aqueous medium. If the volume of the aqueous medium is large, this loss can be significant. In addition, depending upon the type of lipid and biologically active compound present in the liposome, there is the potential for chemical degradation of the lipid components and/or the biologically active components in the aqueous dispersion.

These factors restrict the use of liposomes as practical carriers of biologically active compounds. One solution suggested for overcoming the limited physical stability of liposomes is to prepare and store the lipid/biologically active compound film and then disperse the film to form liposomes just prior to administration. However, unit dose film preparation presents serious practical difficulties in that the containers would require a high surface area to facilitate solvent evaporation and deposition of a thin film suitable for rapid dehydration to form liposomes readily. This type of container by virtue of its bulk would present severe storage problems. Other methods suggested for preparing liposome components in a solid form for storage have included freeze-drying the prepared aqueous liposome suspension as described in U.S. Pat. Nos. 4,229,360 to Schneider, et al. and 4,247,411 to Vanlerberghe and by freeze-drying the liposome components from a suitable organic solvent as described in U.S. Pat. No. 4,311,712 to Evans, et al. These freeze-dried preparations result in a porous matrix of liposome components which is easily hydrated.

It is known that the size of a liposome product could have a bearing on the delivery of a medicament, carried by the liposome product, to the desired site at the desired time. Thus, if the size of a liposome is too small, the liposome carrying the medicament may persist in the circulating plasma for an exceedingly long period and the medicament will not be delivered to the tar-

geted site within the requisite time. If the size of the liposome is too large, the liposome can cause a capillary blockage and/or may be removed by untargeted tissue. Thus, where the medicament carried by the liposome is amphotericin B, if the size of the liposome is larger than desired, the liposome carrying the amphotericin B may be removed by the lungs or spleen as opposed to the desired site, namely, the liver.

One of the problems associated with the preparation of liposomes using the conventional "cast film" method is that usually a heterogeneous population of liposomes with respect to size is normally obtained. A more uniform size population can be obtained by use of ultrasonification of the liposomal material; however this generally results in the formation of liposomes of small size.

BRIEF DESCRIPITON OF THE INVENTION

In accordance with the present invention, a method is provided for preparing thin films of liposome components (also known as proliposomes), which are not subject to the physical stability problems set out above, and which may be employed to form liposome preparations immediately prior to administration.

In addition, the mean size of liposome preparations formed upon hydration of the proliposomes, produced by the method of the present invention will be more readily controlled than is possible by use of other techniques. Thus, it is possible in accordance with the method of the present invention to form the proliposomes in a manner such that the means size of the final hydrated liposome product can be controlled to suit the particular medicament to be carried by the liposome and the particular theraputic application and site.

The method of the present invention includes the steps of forming a solution in an organic solvent of at least one liposome-forming amphipathic lipid, optionally, at least one biologically active compound, and, optionally, at least one adjuvant, and employing the so-formed organic solution to coat a suitable water-soluble particulate carrier material (which carrier material is poorly soluble or insoluble in the organic solvent) to form a thin film of liposomal components (of controlled and desired thickness) on particles of the carrier material. The so-coated carrier material is also referred to as a proliposome.

In addition, in accordance with the present invention, a method is provided for forming a liposome preparation which method includes the step of exposing the water-soluble particulate material coated with the thin film of liposome components to water thereby causing the thin film of liposome components to hydrate and the carrier material to dissolve to give a liposome preparation, similar to that prepared by hydration of cast films with a solution of the carrier material, except that the so-formed liposome will have a desired size distribution.

Further, in accordance with the present invention, there is provided the intermediate formed above, that is the proliposome, which is comprised of the relatively stable particulate water-soluble carrier material coated with a thin film of liposome components (of controlled and desired thickness) and which is useful for forming the liposome preparation.

The problems associated with the physical stability of liposome dispersions on storage may be overcome by forming the aqueous dispersion of the coated powdered carrier material prior to administration. Additionally, the chemical integrity of the biologically active compounds and lipid components may be protected in the coated powdered preparations by the incorporation of antioxidants therein or packing the coated powdered material under inert atmospheres, for example. Moreover, the problems associated with size distribution of final hydrated liposome product as encountered in prior art liposomes' formation are substantially eliminated by forming the proliposomes of the invention in a manner such that the film thickness, load or amount, for example, in mg/m^2 of lipid coated on the water-soluble particulate carrier material is controlled and in this way the final hydrated liposome product of desired mean size is attainable.

DETAILED DESCRIPITON OF THE INVENTION

In carrying out the method of the invention for preparing the particulate water-soluble carrier materials coated with a thin film or load of a predetermined amount of liposomal components, a predetermined amount of at least one liposome forming amphipathic lipid, optionally, at least one biologically active compound, and, optionally, at least one adjuvant are dissolved in an organic solvent and a predetermined amount of this organic solution is used to coat a suitable water-soluble carrier material which is poorly soluble or insoluble in the organic solvent. For low melting point liposome-forming amphipathic lipids (that is have a melting point below 50° C.), the optional biologically active compound and optional adjuvant may be directly dissolved in the lipid and this organic solution used to coat a suitable carrier material.

In general, in carrying out the method of the invention the liposomal components (that is the liposome-forming amphipathic lipid, optional biologically active compound and optional adjuvant) are dissolved in an organic solvent employing a weight ratio of liposomal components: organic solvent of within the range of from about 0.005:1 to about 0.5:1 and preferably from about 0.01:1 to about 0.25:1. Predetermined amounts of the organic solution is then used to coat particles of water-soluble carrier material to form a thin film or coating of such liposomal components on the particles of carrier material, which film will be equivalent to a loading on the particulate carrier of from about 0.5 mg/m^2 to about 100 g/m^2, and preferably from about 5 $mg/^2$ to about 50 g/m^2, depending upon the type of carrier material employed, (including its porosity and surface area) the type of lipid employed (including its content of neutral and/or negatively-charged phospholipid). It has been found that a predetermined degree of loading of the liposomal components on the carrier material, as outlined above, will facilitate ultimate formation of final liposome product of desired size, such as a mean diameter (or mass median volume-equivalent diameter) of within the range of from about 25 nm to about 12 um and preferably from about 100 nm to about 6 um.

It has also been found that the size of the final liposome product may be affected by the amount of neutral or negatively-charged phospholipid employed in forming the proliposomes. Thus, where neutral phospholipid such as egg lecithin and ergosterol are employed, it has been found that employing increasing amounts of such neutral phospholipids may cause increase in size in final liposome product, whereas where negatively-charged phospholipids such as, for example, dimyristoylphosphatidyl glycerol (sodium salt) is employed, it has been

found that employing increasing amounts of such negatively-charged phospholipids may cause decrease in size in final liposome product.

In addition, it has been found that the pH of the hydration medium (over a range of 3 to 8), the ionic strength and the nature of the hydration medium (that is, the presence or absence of various salts for example, NaCl or RbCl) used in forming the final liposome product can have an effect on size of liposome product.

The lipid will be present in the organic solution (which may contain an additional organic solvent), to be used to coat the carrier material, in an amount of within the range of from about 1 to about 25% by weight, depending upon the solubility of the lipid in the solvent or solvent mixture used, and preferably from about 2.5 to about 12.5% by weight of such solution. The optional biologically active compound and optional adjuvant material will be present in the coating solution in varying amounts depending upon the nature of the particular compound and/or material employed.

The ratio of lipid to optional biologically active compound in the coating solution will depend upon the lipid solubility or binding of the biologically active compound used. Thus, the coating to be applied to the carrier material will normally contain a weight ratio of lipid:optional biologically active compound of within the range of from about 5:1 to about 1000:1 and preferably from about 10:1 to about 200:1 depending upon the particular biologically active compound to be employed. For example, where the biologically active compound is an anti-infective, such as an antibiotic or an anti-fungal agent, the lipid will be present in a weight ratio to the biologically active compound of within the range of from about 5:1 to about 1000:1 and preferably from about 10:1 to about 300:1. Where the biologically active compound is a contrast agent, the lipid will be present in a weight ratio to the contrast agent in an amount of within the range of from about 5:1 to about 1000:1 and preferably from about 10:1 to about 200:1.

The amounts of optional adjuvant material and biologically active material employed in the coating will comprise amounts conventionally employed in forming liposomes.

The amount of coating applied to the carrier material will depend upon physical characteristics of the carrier material such as surface area and isotonicity requirements, and the desired degree of loading or film thickness and ultimately the desired size of final liposome product. Thus, the coating will normally be present in a weight ratio to carrier material in an amount of within the range of from about 0.02:1 to about 6:1 and preferably from about 0.2:1 to about 5.5:1.

Any amphipathic lipid which is known to be suitable for preparing liposomes by known methods can be used in the method of this invention. Thus, a wide variety of lipids may be used but non-immunogenic and biodegradable neutral lipids would be preferred. Examples of suitable lipids are the neutral phospholipids, for example, natural lecithins, such as egg lecithin or soya bean lecithin, or synthetic lecithins such as saturated synthetic lecithins, for example, dimyristoylphosphatidyl choline, dimyristoylphophatidyl glycerol (e.g. Na salt), dipalmitoyl phosphatidyl choline or distearoyl phosphatidyl choline or unsaturated synthetic lecithins, such as dioleyl phosphatidyl choline or dilinoleyl phosphatidyl choline, with neutral egg lecithin and soya bean lecithin being preferred.

The biologically active compound employed in the present invention may be any compound of biological interest; for example, the compound may be a medicament, such as an anti-infective, for example, amphotericin B, ketoconazole, isoconazole, and benzyl penicillin, anti-tumor agents, such as 5-fluorouracil, methotrexate, actinomycin D, enzyme, hormone, contrast agent, or marker compound or NMR imaging agent, such as 4-succinyl-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl.

Examples of contrast agents suitable for use in the present invention include, but are not limited to the following: N,N'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[(2-hydroxy-1-oxopropyl)-amino]-2,4,6-triiodo-1,3-benzenedicarboxamide (Bracco 15,000), metrizamide, diatrizoic acid, sodium diatrizoate, meglumine diatrizoate, acetrizoic acid and its soluble cationic salts, diprotrizoic acid and its soluble inorganic and organic cationic salts, iodamide, sodium iodipamide, meglumine iodipamide, iodohippuric acid and its soluble salts, iodomethamic acid and its soluble salts, iodopyracetiodo-2-pyridone-N-acetic acid and its soluble salts, 3,5-diiodo-4-pyridone-N-acetic acid (iodopyracet), 3,5-diiodo-4-pyridone-N-acetic acid diethanolamine salt, iodo-2-pyridone-N-acetic acid and its amine salt, iothalamic acid and its soluble salts, methanesulfonic acid, metrizoic acid and its soluble salts, sodium ipodate, ethiodized oil, iopanoic acid, iocetamic acid, tyropanoate sodium, iopydol, iophenoxic acid, iophendylate, and other chemically related iodinated contrast agents. Unless indicated otherwise, where applicable, the contrast agents which may be employed herein include inorganic, organic and cationic salts of the above contrast agent, such as the potassium salt, calcium salt, lithium salt, arginin salt, cystein salt, glycin salt, glycyl glycin salt, N-methyl glucosamine salt and other non-toxic aliphatic and alicyclic amines employed in preparing water soluble salts. Other X-ray contrast agents which may be employed herein are disclosed in German Offenlegungsschrift DT 2935-195.

The final liposome preparation containing a contrast agent prepared by the method of the invention may be employed as described in U.S. Pat. No. 4,192,859 which is incorporated herein by reference.

Other proteins and drugs available for use herein as optional biologically active compounds include steroids such as hydrocortisone, colchicine, insulin, cyclic AMP and α-thiodeoxyguanosine, chelating agents and cell modifying substances, such as antigens and interferon inducers.

The present invention is particularly useful in the case of lipid-soluble or lipid-bound biologically active compounds (which include some water-soluble compounds, such as proteins).

The method of this invention, like other methods of preparing liposomes, will result in partial incorporation of water-soluble biologically active compounds. Usually the formation of liposomes containing this type of compound is followed by removal of the unencapsulated material; however, in some instances coadministration of unencapsulated and liposomally entrapped biologically-active compounds may be advantageous.

The optional adjuvants suitable for use in the present invention may be:

(a) substances which are known to provide a negative charge on the liposomes, for example, egg phosphatidic acid or dicetyl phosphate;

(b) substances known to provide a positive charge, for example, stearyl amine, or stearyl amine acetate;

(c) substances shown to affect the physical properties of the liposomes in a more desirable way; for example, sterols such as cholesterol, ergosterol, phytosterol, sitosterol, sitosterol pyroglutamate, 7-dehydrocholesterol, lanosterol, or caprolactam, will affect membrane rigidity;

(d) substances known to have antioxidant properties to improve the chemical stability of the particulate carrier coated with liposome components, such as tocopherol, propyl gallate, ascorbyl palmitate, or butylated hydroxy toluene.

Suitable organic solvents for use in dissolving or aiding in dissolution of the above-mentioned mixture of lipid and optionally active compound and optional adjuvant ingredients include, but are not limited to, ethanol, methanol, chloroform, dichloromethane, diethyl ether, carbon tetrachloride, ethyl acetate, dioxane, cyclohexane and the like, with methanol, ethanol or chloroform being preferred.

The carrier material to be coated may be any water-soluble material which is suitable for oral or parenteral use, but is poorly soluble or insoluble in the organic solvent used for dissolving the mixture of lipid, optional active compound and optional adjuvant. Examples of such carrier materials include sodium chloride, lactose, dextrose, and sucrose, with sodium chloride being preferred.

Other examples of carrier material to be coated will include physiologically acceptable free-flowing powder which, even after processing, will remain substantially granular and free-flowing, will have a high water-solubility, for example, in excess of about 10% by weight in water and a rapid dissolution rate in water, for example, complete solution in 3 to 4 minutes at 40° C., is suitable for oral or parenteral use, is poorly soluble or insoluble in the organic solvent, and will preferably form an isotonic solution in water in a concentration range of from about 1 to about 10% w/v, and preferably from about 3 to about 7% w/v; examples of such additional carrier materials include sorbitol, mannitol, xylitol, or naturally occurring amino acids such as arginine or glycine, with sorbitol being preferred.

It may be advantageous to use micronized forms of the carrier materials (that is, having an average particle size of less than about 10 microns) as the high surface area would facilitate the hydration and dissolution of the liposomal components. However, the carrier materials may have an average particle size of up to and in excess of 500 microns and still be useful. The amount of carrier material used may be adjusted so that the final reconstituted suspension is iso-osmotic with the blood, although for small volume injections this may not be necessary. As a suitable aqueous medium for dispersion distilled water, isotonic saline or buffer solution may be used, the temperature of which may be modified to exceed the phase transition temperature of the lipid components used in formulation.

The liposomal components (excluding the biologically active compound) preferably are binary mixtures of lipid such as egg lecithin and a sterol selected from the group listed hereinabove (e.g., ergosterol or cholesterol), or ternary mixtures of such lipid, dicetyl phosphate, or other negatively-charged phospholipid such as dimyritoylphosphatidyl glycerol, and a sterol selected from the group listed hereinabove, in the preferred molar ratios of 7:2:1, respectively. The molar percentage of lipid may range from about 55 to about 95% and the sterol from about 5 to about 35% based on a binary mixture. The molar percentage of lipid may range from about 50% to about 80%, the dicetyl phosphate from 0 to about 30%, and the sterol from about 5 to about 30%, based on a ternary lipid mixture. The lipid is employed to take advantage of its property of swelling in salt solutions. Dicetyl phosphate or dimyristoylphosphatidyl glycerol has the property of imparting a negative charge to the lipid membranes so that the mutual repulsive action of opposing channel surfaces widens the channels.

The components which constitute the liposomal mixture are commercially available or may readily be prepared.

Coating of the carrier material may be achieved by applying organic solutions of liposomal components dissolved either in the lipid phase or in a suitable organic solvent followed by solvent removal. Alternatively, a solution of liposomal components may be spray dried on the carrier material. By suitable containment and sterilization of component materials, sterile carrier material coated with the liposomal components (a proliposome) is produced. The coated carrier material of the invention may be packed in sterile unit dose vials under aseptic conditions and reconstituted immediately prior to use by the physician by hydration of the lipid film (above the phase transition temperature of the lipids) together with dissolution of the particulate carrier material to form a isotonic liposomal suspension.

The final liposome formulations prepared as described above may be administered parenterally, for example, intravenously, as well as orally and topically.

The following Examples represent preferred embodiments of the present invention.

EXAMPLE 1

Five hundred and ten milligrams (510 mg) of dextrose (anhydrous) were placed in a 100 ml round bottom flask and 40 mg of egg lecithin dissolved in 3 ml of chloroform added in 3×1 ml portions. After each portion addition, the solvent was removed under vacuum from the rotating flask. Microscopic examination of the powder showed that the dextrose particles were coated with lipid material.

The so-formed coated carrier may be packaged and stored in sterile unit dose vials under aseptic conditions and reconstituted immediately prior to use.

Distilled water (5 ml) was added to a portion of the above dextrose preparation coated with phospholipid (225 mg) in a vial and the mixture heated to 60°0 C., for one minute, then agitated by hand to achieve the final dispersion. The size distribution of the liposome preparation was log-normal as determined using a Coulter Counter with a mass median volume equivalent diameter of 5.3 μm and a geometric standard deviation of 1.58.

The above liposome preparation is similar to those prepared by hydration of cast films with a solution of the carrier material.

EXAMPLE 2

Egg lecithin (2.0 g), ergosterol (0.5 g) and amphotericin B (50.0 mg) were dissolved in methanol (10 ml). Lactose (13.0 g) was placed in a 250 ml round bottom flask and the above solution added in 2 ml portions. After each portion addition, the solvent was removed under vacuum from the rotating flask. Microscopic

examination of the powder showed that the lactose particles were coated with lipid material.

The so-formed coated carrier may be packaged and stored in sterile unit dose vials under aseptic conditions and reconstituted immediately prior to use by the physician as follows.

Water for injection (10 ml) was added to a portion of the above lactose preparation coated with liposomal components (0.775 g) in a vial and the mixture heated to about 70° C. in a water bath to aid dissolution of the carrier material. Shaking of the vial caused the preparation to disperse resulting in a milky dispersion. The size distribution of the liposomes formed was log-normal as determined using a Coulter counter with a mass median volume equivalent diameter of 2.5 μm and a geometric standard deviation of 1.56.

The liposome preparation containing the amphotericin B is similar to those prepared by hydration of cast films with a solution of the carrier material.

EXAMPLE 3

Five hundred and ten milligrams (510 mg) of dextrose (anhydrous) were placed in a 100 ml round bottom flask and 40 mg of egg lecithin dissolved in 3 ml of chloroform added in 3×1 ml portions. After each portion addition, the solvent was removed under vacuum from the rotating flask. Microscopic examination of the powder showed that the dextrose particles were coated with lipid material.

The so-formed coated carrier may be packaged and stored in sterile unit dose vials under aseptic conditions and reconstituted immediately prior to use.

Distilled water (5 ml) was added to a portion of the above dextrose preparation coated with phospholipid (225 mg) in a vial and the mixture heated to 60° C. for one minute, then agitated by hand to achieve the final dispersion. The size distribution of the liposome preparation was log-normal as determined using a Coulter Counter with a mass median volume equivalent diameter of 5.3 μm and a geometric standard deviation of 1.58.

The above liposome preparation is similar to those prepared by hydration of cast films with a solution of the carrier material.

EXAMPLE 4

Egg lecithin (2.0 g), ergosterol (0.5 g) and amphotericin B (50.0 mg) were dissolved in methanol (10 ml). Lactose (13.0 g) was placed in a 250 ml round bottom flask and the above solution added in 2 ml portions. After each portion addition, the solvent was removed under vacuum from the rotating flask. Microscopic examination of the powder showed that the lactose particles were coated with lipid material.

The so-formed coated carrier may be packaged and stored in sterile unit dose vials under aseptic conditions and reconstituted immediately prior to use by the physician as follows.

Water for injection (10 ml) was added to a portion of the above lactose preparation coated with liposomal components (0.775 g) in a vial and the mixture heated to about 60°–70° C. in a water bath to aid dissolution of the carrier material. Shaking of the vial caused the preparation to disperse resulting in a milky dispersion. The size distribution of the liposomes formed was log-normal as determined using a Coulter Counter with a mass median volume equivalent diameter of 2.5 μm and a geometric standard deviation of 1.56.

The liposome preparation containing the amphotericin B is similar to those prepared by hydration of cast films with a solution of the carrier material.

EXAMPLE 5

Egg lecithin (89 mg), cholesterol (24 mg), dicetyl phosphate (12.4 mg) and amphotericin B (2.6 mg) were dissolved in methanol (10 ml). Sorbitol (500 mg) was placed in a 250 ml round bottom flask and the above solution added in 2 ml portions. After each portion addition, the solvent was removed under vacuum from the rotating flask. Microscopic examination of the powder showed that the sorbitol particles were coated with lipid material.

The so-formed coated carrier may be packaged and stored in sterile unit dose vials under aseptic conditions and reconstituted immediately prior to use by the physician as follows.

Water for injection (10 ml) was added to a portion of the above sorbitol preparation coated with liposomal components (0.628 g) in a vial and the mixture heated at 60°–70° C. to dissolve the carrier material and form a liposome preparation.

The liposome preparation containing the amphotericin B is similar to those prepared by hydration of cast films with a solution of the carrier material.

EXAMPLE 6

Egg lecithin (121 mg), ergosterol (5 mg) and amphotericin B (5 mg) were dissolved in methanol (10 ml). Sorbitol (500 mg) was placed in a 250 ml round bottom flask and the above solution added in 2 ml portions. After each portion addition, the solvent was removed under vacuum from the rotating flask. Microscopic examination of the powder showed that the sorbitol particles were coated with lipid material.

The so-formed coated carrier may be packaged and stored in sterile unit dose vials under aseptic conditions and reconstituted immediately prior to use by the physician as follows.

Water for injection (10 ml) was added to a portion of the above sorbitol preparation coated with liposomal components (0.631 g) in a vial and the mixture heated at 60°–70° C. to dissolve the carrier material and form a liposome preparation.

The liposome preparation containing the amphotericin B is similar to those prepared by hydration of cast films with a solution of the carrier material.

EXAMPLE 7

Determination of Effect of Negatively-charged Phospholipids on Size of Liposomes Derived from Proliposomes

A. Preparation of amphotericin B (AmB)/lipid/sterol) in organic solutions

A solution/suspension of amphotericin B in methanol was prepared at 1 mg/ml; extended shaking/sonication was required to achieve adequate levels of drug in solution. Undissolved material was removed by passing the suspension through a 0.2 μm filter (Millipore GVWP) Amphotericin B concentration of the filtrate was determined spectrophotometrically. In general, a final concentration of about 0.8 mg/ml was obtained. Dimyristoylphosphatidyl chlorine (DMPC) and dimyristoylphosphatidyl glycerol (Na salt) (DMPG) (molar ratio=7:3) were dissolved in chloroform such that the total weight of lipid was 16-fold greater than the total weight

of amphotericin B in solution. This phospholipid:drug ratio was chosen to avoid saturation of the lipid bilayers with amphotericin B. Saturation of the bilayers with a water-insoluble drug may be manifested by the presence of extra-liposomal crystalline material, often observable by light microscopy. Thus, for this formulation, an encapsulation efficiency approaching 100% was obtained. The volume of chloroform utilized was adjusted so that the final volume ratio of methanol to chloroform was 2:1.

B. Egg lecithin/ergosterol/amphotericin B organic solution

In general, a similar procedure to the one outlined above was utilized except ergosterol and egg lecithin were dissolved in the methanolic solution of amphotericin B (chloroform was omitted). A molar ratio of 28:2.3:1 (egg lecithin:ergosterol:amphotericin B) was utilized throughout.

C. Preparation of Proliposomes

(1) Sorbitol-based proliposome

In a typical batch, 10 g sorbitol (total pore area of 125–500 μm cut was 33.1 m^2/g) was placed in a 100 ml round-bottomed flask. A modified rotary evaporator (Büchi Rotavapor-R) was evacuated (93–101 kPa) and the rotating flask lowered into a water bath at 35°–45° C. (dependent upon the nature of the lipid and carrier). A weight of the lipid/amphotericin B/(sterol) solution equivalent to half the weight of sorbitol in the flask was introduced into the tumbling powder bed via the feed line running through the condenser unit. Evaporation was allowed to proceed until the powder bed temperature reached about 15° C. and began to rise at which point a second aliquot of solution was introduced. This process was repeated until all the solution had been deposited onto the sorbitol. At no time was the powder bed allowed to become over-wet (such that a slurry was formed), nor was the unit operated in the absence of a vacuum; failure to observe these precautions resulted in complete dissolution of the sorbitol. After addition of the final aliquot, evaporation was continued until the powder bed temperature began to rise substantially (to about 30° C.). At this point, the material was removed from the flask and passed through a 950 μm aperature stainless steel sieve. The material was then dried overnight in a desiccator in vacuo at room temperature. After drying, material <75 μm and >600 μm was removed by sieving and was discarded; the remaining material was packaged under nitrogen in unit dose vials such that upon hydration with water an isotonic solution would be formed (i.e., equivalent to 5% w/v sorbitol).

The loading of liposomal components onto sorbitol was approximately 7.9 mg/m^2.

Differential scanning calorimetry (employing DuPont Instruments Model 910 in conjunction with a Dupont 990 Computer/Thermal Analyzer) of egg lecithin-/ergosterol/AmB proliposomes revealed the phospholipid endotherm to give an extrapolated onset temperature of −14.1° C. with a phase transition temperature (Tm) of −6.8° C. Thus, proliposomes of this formulation were hydrated at room temperature (20° C.), substantially above the Tm of the phospholipid. In contrast, proliposomes composed of DMPC and DMPG (Tm=23° C.) required hydration above 30° C. (preferably 37° C.). Differential scanning calorimetry of this formulation revealed a broadening of the phospholipid endotherm (by comparison with pure phospholipids) resulting in an extrapolated onset temperature of 20.6° C.

(2) Sodium chloride-based proliposomes

The procedure adopted was essentially the same as for sorbitol-based proliposomes, with the following exceptions: first, in a typical batch, 1.8 g sodium chloride was used (rather than 10 g sorbitol) so that after addition of the appropriate amount of water, the resulting liposome suspension would be iso-tonic. Second, the reduction in carrier weight necessitated a reduction in the volume of lipid/AmB solution added during manufacture. Third, a weight ratio of sodium chloride to total lipid of 1:5.4 was found optimal.

D. Results

(1) Sorbitol-based Proliposomes Composed of Lipid/sterol/AmB

Scanning electron micrographs of both uncoated sorbitol and sorbitol coated with egg lecithin/ergosterol/AmB show that the porous structure of the sorbitol appeared to be maintained after proliposome manufacture, thus suggesting that the majority of the lipid-/ergosterol/AmB was within the matrix of the granules.

Upon hydration at room temperature, sorbitol-based proliposomes composed of egg lecithin/ergosterol-/AmB formed multilamellar liposomes with a mass median volume equivalent diameter of approximately 1.8 μm as determined by Coulter Counter; this size was a consistent feature of the formulation (when manufactured under defined conditions).

(2) Sorbitol-based Proliposomes Composed of DMPC/DMPG/AmB (molar ratio=7:3:0.52) hydrated (at 37° C.) to form liposomes with a mean diameter of 120 nm (polydispersity=0.31) (analysis by photon correlation spectroscopy (PCS), employing a Malvern 4600M with a 35 mW helium-neon laser (Spectra Physics Model 124B)) substantially smaller than those composed of egg lecithin. Since the extent of loading onto carrier was the same for both formulation, one reason for the difference in particle size was thought to be due to the presence of charged phospholipid (DMPG) in the DMPC/DMPG/AmB formulation. To test this hypothesis, a variety of proliposomal formulations were prepared consisting of various DMPC:DMPG ratios (at a constant lipid to sorbitol ratio). Upon hydration at 37° C., liposome particle size was measured by Coulter Counter. Results are presented in Table I set out below and indicate that as the level of the negatively charged lipid increases, so mean liposome size decreases. Thus, at a constant lipid to sorbitol loading, the size of liposomes derived from proliposomes may be modified by the content of negatively-charged phospholipid.

TABLE I

EFFECT OF DMPC:DMPG RATIO ON THE SIZE OF LIPOSOMES DERIVED FROM PROLIPOSOMES (AS DETERMINED BY COULTER COUNTER ANALYSIS)

Molar Ratio DMPC:DMPG	Mass Median Volume Equivalent Diameter (μm ± SD)
25:1	1.5 ± 1.4
50:1	3.0 ± 2.8
100:1	3.5 ± 2.4

TABLE 1-continued

EFFECT OF DMPC:DMPG RATIO ON THE SIZE OF LIPOSOMES DERIVED FROM PROLIPOSOMES (AS DETERMINED BY COULTER COUNTER ANALYSIS)

Molar Ratio DMPC:DMPG	Mass Median Volume Equivalent Diameter (μm ± SD)
250:1	4.9 ± 4.1

One of the simplest and most convenient procedures for preparing liposomes is that of casting a dry lipid film on the inside of a round-bottomed flask, by evaporation from organic solvent. Subsequent hydration of dry lipid by aqueous phase (above the phase transition temperature of the lipid) results in the formation of liposomes. Early experiments had indicated that the size of liposomes derived from DMPC/DMPG/AmB (7:3:0.52) cast films deposited on the inside wall of a round-bottomed flask (at a loading of about 30 g/m^2) had typical mass median volume equivalent diameters of approximately 2.0 μm. In contrast, liposomes derived from sorbitol-based proliposomes of similar formulation (at a loading of 7.9 mg/m^2) had mean sizes of 100–150 nm.

The approach taken therefore, was to determine whether an increase in lipid film thickness on a proliposome carrier results in an increase in mean liposome size. To achieve this aim, a relatively non-porous carrier, namely, sodium chloride, was utilized. The surface area of sodium chloride 315–600 μm size cut was determined to be 0.12 m^2/g. DMPC/DMPG/AmB were coated onto the carrier to a loading of about 47 g/m^2 (comparable to that of the conventional cast film procedure). Hydration of the sodium chloride-based proliposomes with water at 37° C. resulted in the formation of liposomes of similar mass median volume equivalent diameter to those produced by the conventional cast film method (i.e., approximately 1.8 μm).

To eliminate the possibility that sodium chloride per se was responsible for the substantial increase in liposome size, sorbitol based proliposomes were hydrated in normal saline while cast lipid films were hydrated in 5% w/v sorbitol. While minor changes in particle size were seen, nothing as substantial as that displayed by altering lipid film thickness was observed. Thus, a degree of control over liposome size could be obtained by considering the nature of the carrier, the type of lipid and its final degree of loading.

Further, marked differences in liposome size could be achieved with the same liposome formulation but with differences in lipid loading (the nature of the different carrier materials had only a small effect on liposome size in this study).

With the egg lecithin/ergosterol/amphotericin B formulation, further evidence of the importance of lipid film thickness was obtained by sampling the proliposome batch at various points in the process. Thus, a mass median volume equivalent diameter of 3.1 μm was attained at 75% of the optimal loading, whereas a mean size of 4.25 μm was achieved at 100% loading.

EXAMPLE 8

Proliposomes were prepared as described in Example 7 on particulate sorbitol sieved to obtain a 125–500 μm size cut. The sorbitol was coated with phospholipids/-sterol/drug, that is, DMPC:DMPG:Erg:AmB.

(1) Determination of Effect of pH of Hydration Liquid on Liposome Size

The effect of pH on the hydrated liposome product may have an effect on liposome size with subsequent effects on tissue distribution and efficacy. As amphotericin proliposomes contain no buffer or other pH controlling material and are hydrated with water for injection (with pH limits of 5.0–7.0 in USP XXI), size variation of the resultant liposomes could be produced if pH has an effect. Proliposomes were hydrated in constant ionic strength buffers over the range pH 3–8 for evaluation of pH of the hydration medium on the size of liposomes produced (Table II).

TABLE I

EFFECT OF HYDRATION MEDIUM pH ON THE SIZE OF LIPOSOMES DERIVED FROM DMPC/DMPG/ERG/AmB PROLIPOSOMES

pH	Mean Liposome Size (μm)
3	1.2
4	0.2
5	~0.25

At pH 3.0 very large liposomes appeared to form but much smaller liposomes occurred at pH 4.0 and above, with some slight changes over the range pH 4.0–8.0. The effect at pH 3.0 may have been due to protonation of the acidic function of DMPG, resulting in destabilisation of the liposome structure. An effect of charge may have been involved. The effect of protonation of DMPG is to reduce the charge on the liposomes and this may be responsible for the increase in liposome size at low pH values.

The increase in size was noted over the range pH 4.0–6.0. This may have been due to increase in amounts of available sodium counter ion from the buffer, as its sodium content increases with increasing pH. The effect is explained in detail below when considering ionic strength. An increase in size between pH 6.0 and pH 8.0 possibly relates to charge changes on the liposomes resulting from deprotonation of other species.

(2) Determination of Ionic Strength on Liposome Size

Further to the pH studies the effect of increasing the ionic strength by addition of salts to a constant pH system was evaluated. This would be important if hydration with media other than water for injection was employed and varied volumes of hydration media were used. As can be seen from the sodium chloride data (Table III), there is an increase in liposome size as the amount of sodium chloride in the hydration medium increases. This can be explained in terms of the sodium ions acting as counter ions to the negatively charged phosphatidylglycerol in the liposomes resulting in a reduction of the net charge on the liposomes.

TABLE III

EFFECT OF HYDRATION MEDIUM COMPOSITION/IONIC STRENGTH ON THE SIZE OF LIPOSOMES DERIVED FROM DMPC/DMPG/Erg/AmB PROLIPOSOMES

NaCl	
Ionic Strength (M)	Mean Liposome Size
~0.04	0.3
~0.08	0.34
0.15	0.32
0.3	0.43
RbCl	

TABLE III-continued

EFFECT OF HYDRATION MEDIUM COMPOSITION/IONIC STRENGTH ON THE SIZE OF LIPOSOMES DERIVED FROM DMPC/DMPG/Erg/AmB PROLIPOSOMES

Ionic Strength	Mean Liposome Size
~0.04	0.16
~0.08	0.21
0.15	0.2
0.3	0.25

As seen in Table III, regarding rubidium chloride, the liposome size effect for a given amount of salt is lower than for sodium chloride suggesting that a higher net charge remains on the liposomes. As the hydrated rubidium ion is very much larger than the sodium ion there may be steric effects preventing it accessing as many potential binding sites within the bilayers of the liposomes. This would result in a higher net charge on the liposome than in the sodium chloride case and thus give rise to smaller liposomes.

As seen from the above results, marked changes in liposome size can be induced by changes in the pH of the hydration medium (over the pH range 3–8). However, with the pH limits set for water for injection (pH 5.0–7.0) little effect of pH on liposome size was noted. Ionic strength and nature of the hydration medium can have a marked effect on liposome size.

EXAMPLE 9

The following was carried out to illustrate the effect that the amount of loading or film thickness of lipid, adjuvant and medicament deposited on a particulate carrier (proliposomes) has on the size of liposomes derived from such proliposomes.

Proliposomes were prepared in a manner similar to that described in Example 7 Parts B and C(1). A methanol solution (400 ml) containing egg lecithin (4.84 g), ergosterol (0.2 g) and amphotericin B (0.2 g) was prepared.

Sorbitol (20 g, 125–500 μm size cut) was placed in a round-bottom flask. A modified rotary evaporator was evacuated and the rotating flask lowered into a water bath at ~40° C. A portion of the above organic solution (10%, that is, 40 ml) was sprayed onto the heated tumbling powder bed in vacuo. After drying, a portion of the proliposomal material was removed for particle size analysis of resultant liposomes.

Further aliquots of methanolic solution were then sprayed onto the remaining powder bed so that loadings of 25, 50, 75 and 100% were achieved; samples of proliposomal material were removed at each stage for size analysis.

Due account was taken for the material removed at each stage by reducing the amount of added methanolic solution accordingly.

Each of the proliposomal materials (10, 25, 50, 75 and 100% loading) was then hydrated by mixing with distilled water at 20°–25° C. in a manner similar to that described in Example 1. The mass median volume-equivalent diameters (microns) of the liposomes so produced were then determined. The % loading or relative film thickness (for each proliposome material) and the size of the liposomes derived from each of such proliposome materials is set out in Table IV.

TABLE IV

% Loading	Mass medium volume Equivalent diameter (microns)
10	1.6 ± 1
25	2.0 ± 1.2
50	2.5 ± 1.4
75	3.2 ± 1.5
100	5.3 ± 2

As can be seen from the results set out in Table IV, where neutral lipids such as egg lecithin (and presumably other neutral lipid formulation, such as egg lecithin and cholesterol are employed), the mean liposome size increases with increase in lipid loading.

From the above results, it is clear that size of the final liposome preparation is dependent upon the loading or relative film thickness of the proliposome. Thus, size of the final liposome may be controlled by controlling the loading or relative film thickness of the proliposome. However, as seen in Example 7, as the level of negatively-charged phospholipids increases, the size of liposomes derived therefrom decreases.

What is claimed is:

1. A method for preparing a stable liposome precursor in the form of a thin film of liposomal components of predetermined amount coated on a water-soluble particulate carrier material which is suitable for intravenous use, which liposome precursor may be hydrated to form a liposome product of controlled and acceptable mean size, which comprises forming a solution, in an organic solvent, of at least one liposome-forming lipid selected from the group consisting of a phospholipid, a natural lecithin and a synthetic lecithin in an amount of from about 1 to about 25% by weight of the organic solution, optionally, at least one biologically active compound which is medicament, protein, enzyme, hormone or diagnostic agent, and, optionally, at least one adjuvant which imparts advantageous properties to the final liposome preparation, and coating a particulate water-soluble carrier material which is suitable for intravenous use and is substantially insoluble in said organic solvent, with the so-formed organic solution to form a thin film of liposomal components of predetermined desired amount on said carrier material which thin film contains a ratio of lipid:optional biologically active compound (where present) of within the range of from about 0.5:1 to about 1000:1, which so-coated particulate water-soluble carrier material may be exposed to a hydration medium to form a final hydrated liposome product of controlled and desired mean size.

2. The method as defined in claim 1 wherein the thin film of liposomal components, coated on the particulate carrier is present in an amount within the range of from about 0.5 mg/m^2 to about 100 g/m^2 of carrier material so that the final hydrated liposome product will have a desired mean size distribution of within the range of from about 25 nm to about 12 μm.

3. The method as defined in claim 1 wherein the organic solution is formed by dissolving said lipid, optionally, said biologically active compound and, optionally, said adjuvant in one or more organic solvents.

4. The method as defined in claim 1 wherein the organic solution is formed by dissolving the optional biologically active compound and optional adjuvant in said lipid, said lipid being of the low melting point liposome-forming type.

5. The method as defined in claim 1 wherein the particulate carrier material is coated with said solution by suspending the carrier material in the solution of liposomal components and spray drying the coated carrier material.

6. The method as defined in claim 1 wherein the lipid is a phospholipid.

7. The method as defined in claim 5 wherein the phospholipid is a natural or synthetic lecithin.

8. The method as defined in claim 1 wherein the lipid is dimyristoylphosphatidyl choline, alone or in combination with dimyristoylphosphatidyl glycerol.

9. The method as defined in claim 1 wherein said solution includes a biologically active compound which is a medicament, contrast agent, enzyme, hormone, or marker compound.

10. The method as defined in claim 1 wherein the adjuvant is egg phosphatidic acid, dicetyl phosphate, or stearyl amine.

11. The method as defined in claim 1 wherein the adjuvant is a sterol.

12. The method as defined in claim 8 wherein the adjuvant also includes a sterol selected from the group consisting of cholesterol, phytosterol, ergosterol, sitosterol, sitosterol, 7-dehydrocholesterol or lanosterol.

13. The method as defined in claim 1 wherein said carrier is sodium chloride, lactose, dextrose, or sucrose.

14. The method as defined in claim 1 wherein said carrier material has a watersolubility in excess of 10% by weight, a rapid dissolution rate in water, and will form an isotonic solution in water in a concentration of from about 1 to about 10% w/v.

15. The method as defined in claim 3 wherein said carrier material is sorbitol, mannitol, or xylitol or a naturally occurring amino acid.

16. The method as defined in claim 1 wherein the carrier material is sorbitol or sodium chloride.

17. The method as defined in claim 1 wherein the organic solvent is ethanol, methanol, chloroform, dichloromethane, diethyl ether, carbon tetrachloride, ethyl acetate, dioxane or cyclohexane.

18. The method as defined in claim 14 wherein the solvent is methanol, ethanol or chloroform.

19. The method as defined in claim 1 wherein the biologically active compound is amphotericin B.

20. The method as defined in claim 1 wherein the lipid is egg lecithin, dimyristoylphosphatidyl choline alone or in admixture with dimyristoylphosphatidyl glycerol, the optional adjuvant is ergosterol, cholesterol or dicetyl phosphate, and the carrier material is sorbitol or sodium chloride.

21. The method as defined in claim 20 wherein the lipid is egg lecithin, and the adjuvant is ergosterol.

22. A stable liposome precursor which when mixed with water forms a liposome preparation of controlled and acceptable mean size distribution, comprising a water-soluble particulate carrier material suitable for intravenous use coated with a predetermined amount of a thin film of liposomal components comprised of at least one liposome-forming lipid selected from the group consisting of a phospholipid, a natural lecithin and a synthetic lecithin, optionally, at least one biologically active compound which is a medicament, protein, enzyme, hormone or diagnostic agent, and, optionally, at least one adjuvant which imparts advantageous properties to the final liposome preparation and which thin film contains a ratio of lipid:optional biologically active compound (where present) of within the range of from about 0.5:1 to about 1000:1.

23. The stable liposome precursor as defined in claim 21 wherein the thin film of liposomal components is present in an amount within the range of from about 0.5 mg/m^2 to about 100 g/m^2 of carrier material so that the final hydrated liposome product will have a desired mean size distribution of within the range of from about 25 nm to about 12 μm.

24. A stable liposome precursor which when mixed with water forms a liposome preparation of controlled and acceptable mean size, comprising a water-soluble particulate carrier material suitable for intravenous coated with a predetermined amount of a thin film comprised of at least one liposome-forming lipid selected from the group consisting of a phospholipid, a natural lecithin and a synthetic lecithin, optionally, at least one biologically active compound which is a medicament, protein, enzyme, hormone or diagnostic agent, and, optionally, at least one adjuvant which imparts advantageous properties to the final liposome preparation, prepared by the method as defined in claim 1.

25. The method as defined in claim 21 including a biologically active compound which is amphotericin B.

26. The stable liposome precursor as defined in claim 22 including a biologically active compound.

27. The stable liposome precursor as defined in claim 26 wherein the biologically active compound is amphotericin B.

28. The stable liposome precursor as defined in claim 22 wherein the lipid is egg lecithin, the adjuvant is ergosterol and the biologically active compound is amphotericin B.

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TAB E

LIPPINCOTT WILLIAMS & WILKINS

INVESTIGATIVE RADIOLOGY
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Biodistribution and Computed tomography Blood-Pool Imaging Properties of Polyethylene Glycol-Coated Iopromide-Carrying Liposomes

[Original Investigations]

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Outline

Graphics

Abstract^

RATIONALE AND OBJECTIVES: Surface-modified contrast-carrying liposomes potentially are useful as computed tomography(CT) blood-pool agents. The biodistribution and CT-imaging behavior of conventional as well as polyethylene glycol (PEG)-coated iopromide-carrying liposomes were tested. Two different types of PEG-ylated lipids were used to demonstrate possible differences.

METHODS: Iopromide-containing liposomes were prepared by a continuous high-pressure extrusion method and subsequently PEG-ylated by simple mixing with either DSPE-PEG2000 or CHHS-PEG2000. The resulting liposomes were investigated in rats (biodistribution) and rabbits(imaging).

RESULTS: Surface modification with CHHS-PEG consistently resulted in less effective stabilization of liposomes in the blood than with DSPE-PEG. In the biodistribution study, no significant differences in blood concentration could be found 1 hour after injection between the different formulations at a dose of 250 mg total iodine/kg body weight(approximately 500 mg lipid/kg). At this dose, the unmodified as well as the DSPE-PEG liposomes displayed prolonged blood circulation with CT density differences above 70 Hounsfield units (aorta) for up to 20 minutes (n = 1).

CONCLUSIONS: DSPE-PEG-coated and unmodified liposomes proved to be useful for CT blood-pool imaging displaying favorable imaging properties. Future studies will have to demonstrate whether PEG-ylation offers diagnostic or toxicologic advantages over conventional vesicles in this indication.

Liposomes have been used successfully as experimental carriers for contrast agents in computed tomography(CT), magnetic resonance (MR) imaging, and nuclear medicine.[1,2] In the past, radiologic application of conventional liposomes was confined to their use in liver and spleen imaging due to the rapid uptake of such vesicles by the cells of the mononuclear phagocytic system (MPS) after intravenous injection.[3]

Recently, however, surface-modified liposomes were shown to be able to avoid the MPS, thus allowing the targeting to non-MPS organs. Especially the inclusion of lipid derivatives of polyethylene glycol (PEG) in the liposome membrane has been shown to be very potent in increasing liposomal circulation times.[4] It has been suggested that contrast-carrying liposomes with extended lifetimes in the circulation might be useful as blood-pool (vascular) imaging agents, especially in MR imaging and scintigraphy.[5] Blood-pool imaging would be of special interest for the evaluation of the current state of the blood flow and for the discovery of irregularities caused by pathologic changes.[6] Thus, it could be demonstrated that DPPE-PEG-coated ^{99m}technetium-labeled large unilamellar vesicles (200 nm) exhibit a prolonged blood residence in rabbits, which might render them useful as a vascular marker in various nuclear diagnostic procedures.[7] As far as the use of liposomal CT contrast agents in blood-pool indications is concerned, no specific studies have been reported thus far. In an early study with conventional(unmodified) diatrizoate liposomes, however, a prolonged blood-pool residence already was found.[8]

In the present study, conventional and surface-modified liposomes containing the nonionic monomeric contrast agent iopromide (Ultravist) were tested as potential blood-pool agents for CT. The impact of two different coating agents (DSPE-PEG2000 and CHHS-PEG2000) on the plasma stability and biodistribution in rats was examined. Additionally, the CT imaging properties of these liposomes were investigated in an orientating study in rabbits.

Materials and Methods^

Preparation and Characterization of Iopromide-Carrying Liposomes^

The iopromide liposomes (original iodine concentration 60 mg/g total suspension) were prepared by the continuous high-pressure extrusion method using 0.2-μm filters for the last extrusion step.9 Subsequent PEG-coating was performed by simple mixing of the preformed liposomes made from soy phosphatidylcholine (SPC), cholesterol(CH), and soy phosphatidylglycerol (SPG) (SPC/CH/SPG 6:3:1 molar ratio, original lipid concentration 120 mg/g total suspension) with 5 mol% of either DSPE-PEG2000 or CHHS-PEG2000 overnight (16 hours at room-temperature on a magnetic stirrer).10

Encapsulation efficiency was determined by equilibrium dialysis and liposome size by photon correlation spectroscopy as described before.9 The zeta potential of the liposomes was calculated from the results of electrophoretic mobility measurements (20 seconds at 25°C) on a Zetasizer IIc (Malvern Ltd, Malvern, UK).10

Plasma Stability^

Leakage of encapsulated iopromide from liposomes in human plasma (DRK-Blutspendedienst, Berlin, Germany) was investigated at 37°C by equilibrium dialysis.11 Encapsulation was determined after 1, 2, 4, and 6 hours of dialysis against plasma and related to the encapsulation efficiency obtained after dialysis against 300 mM mannitol solution over 1 hour (100% value).

Biodistribution Study in Rats^

The experiments were performed in male rats (Wistar strain, 136-160 g body weight). The biodistribution was studied over a period of 24 hours after administration of approximately 4 mL/kg (250 mg total iodine/kg = approximately 500 mg lipid/kg) into a tail vein at a rate of 0.5 mL/minute. At different time points (15 minutes, 60 minutes, 4 hours, and 24 hours after injection) four rats were killed under ether anesthesia by exsanguination from the vena cava. The iodine concentration in the different organs was determined as described previously.12

Computed Tomography Study in Rabbits^

Male rabbits (Hare-rabbits) weighing 2.7 to 3.0 kg were obtained from a breeder. At the beginning of the imaging study, the animals were anesthetized by subcutaneous administration of a mixture of xylazine (10 mg/kg) and ketamine (25 mg/kg). The animals were intubated and ventilated with room air. Before ventilation spontaneous respiration was inhibited through intravenous administration of repeated doses of suxamethonium chloride.

The liposomes or the free contrast agent iopromide(Ultravist) were injected manually at a dose of 250 mg total iodine/kg via an ear vein as a bolus. One animal per preparation was imaged by CT (Somatom Plus S, Siemens, Germany) (120 kV, 165 mA, 8-mm slice thickness, scanning time 1 second), taking scans while the respirator was turned off.

Data Analysis^

Statistical analysis of the plasma stability and biodistribution data was performed by means of Student's t test($P < 0.05$). The density differences obtained in the CT imaging study in various organs were listed as [DELTA] Hounsfield unit ([DELTA]HU) (postcontrast minus precontrast density). Curve fitting of the CT-imaging data (density differences in aorta) was performed by iterative nonlinear regression using Excel (Microsoft Corporation, Seattle, WA).

Results^

Liposome Properties^

Iopromide-carrying liposomes (SPC/CH/SPG 6:3:1) were prepared by the continuous high-pressure extrusion method and used without prior removal of the unencapsulated contrast agent. Subsequently, surface-modification was performed by simple mixing with the respective PEG-derivative overnight. In the case of DSPE-PEG this procedure was accompanied by a drastic increase in vesicle size. Thus, the resulting mean diameter amounted to 204 nm compared with 132 nm for the unmodified and 149 nm for the CHHS-PEG liposomes. As shown in Table 1, encapsulation was not affected noticeably by the PEG-coating procedure ranging from approximately 43% to 45%.

TABLE 1. Physicochemical Properties of Surface-Modified Iopromide Liposomes(n = 1)

The zeta potential of the negatively charged (SPG) unmodified liposomes, which originally amounted to -44.5 mV, was considerably reduced by the surface-modification. The resulting zeta potentials amounted to - 12.2 mV (CHHS-PEG) and - 12.0 mV (DSPE-PEG). As shown by cryo-electron microscopy (Fig. 1), the liposomes remained intact during the PEG-coating procedure (CHHS-PEG), displaying mainly unilamellar characteristics.

Figure 1. Cryo-electron micrograph of CHHS-polyethylene glycol liposomes.

Plasma Stability^

The stability of the unmodified and modified iopromide liposomes in human plasma was determined by equilibrium dialysis of a liposome/plasma mixture against the respective plasma over a period of up to 6 hours (Fig. 2). As can be seen, the relative encapsulation of the unmodified liposomes dropped to approximately 74% and 62% after 1 and 2 hours, respectively. The DSPE-PEG liposomes displayed the highest stability compared with the other formulations over the whole study period with 1- and 2-hour values as high as 84% and 79%, respectively. However, the slight differences in plasma stability observed between the tested preparations after 1 hour were shown to be statistically insignificant (Fig. 2).

Figure 2. In vitro stability of unmodified and modified iopromide liposomes in human plasma.

Biodistribution in Rats^

The biodistribution of the PEG-coated versus unmodified(conventional) liposomes was studied in rats at a dose of 250 mg total (encapsulated plus unencapsulated) iodine/kg body weight (approximately 500 mg total lipid/kg). During the first hour after administration, no significant differences were seen with blood concentrations around approximately 1.4 mg iodine/g wet weight (36% of dose) and 1.2 mg iodine/ g (30% of dose) after 15 minutes and 1 hour, respectively (Fig. 3A). Starting at 4 hours after injection, however, the DSPE-PEG liposomes displayed a significantly higher blood level compared with the other liposome formulations amounting to approximately 28% of dose (1.1 mg iodine/g wet weight). The blood concentration of the CHHS-PEG liposomes was only insignificantly higher than that of the unmodified liposomes at 4 hours after injection (20% versus 16% of dose). After 24 hours, the CHHS-PEG liposomes unexpectedly displayed the significantly lowest blood level of all tested preparations.

Figures 3A-3C. Biodistribution of unmodified and modified iopromide liposomes in (A) blood, (B) liver, and (C) spleen of rats at a dose of 250 mg total iodine/kg body weight. Data are mean values of four animals.

Over the 24-hour investigation period the maximum liver concentration of the tested liposomes was always lower than 0.4 mg iodine/g or 7% of dose (Fig. 3B). Surprisingly, the CHHS-PEG liposomes consistently displayed the highest liver uptake (significant at 4 and 24 hours after injection). After 24 hours, the unmodified vesicles showed the significantly lowest liver concentration with approximately 0.1 mg iodine/g wet weight (1.5% of dose).

In the spleen, an almost constant increase in iodine concentration was seen for all formulations with the unmodified liposomes consistently showing the lowest uptake (significant at 1 and 4 hours after injection, Fig. 3C). The surface-modified liposomes showed a parallel increase in spleen uptake during the first 4 hours after injection. After 24 hours, however, the accumulation of the DSPE-PEG liposomes had dropped (1.0 mg iodine/g or 2.2% of dose) while that of the CHHS-PEG liposomes had further increased to the significantly highest level (2.4 mg iodine/g or 4.9% of dose).

Computed Tomography Study in Rabbits^

During the first 30 seconds after the start of the injection of 250 mg total iodine/kg body weight (bolus = 15 to 20 seconds) densities up to 700 [DELTA]HU could be measured in the aorta (data not shown). Two minutes after administration blood densities ranged from approximately 70 [DELTA]HU (CHHS-PEG) to 90 [DELTA]HU (DSPE-PEG) (Fig. 4). The CHHS-PEG liposomes consistently gave the lowest blood enhancement with a 1-hour value of approximately 45 [DELTA]HU compared with 65 [DELTA]HU for the DSPE-PEG liposomes. The unmodified liposomes resulted in the highest liver density over the whole observation period reaching a plateau around 40 [DELTA]HU approximately 2 minutes after injection (data not shown).

Figure 4. Computed tomography blood densities (aorta) in rabbits at a dose of 250 mg iodine/kg body weight. Data represent the results of one animal for each preparation.

In the rabbit that received the unmodified liposomes a bright enhancement of the aorta (155 [DELTA]HU) and some opacification of the liver vessels already was seen 10 seconds after injection (Fig. 5B). Only 5 seconds later the liver vasculature was enhanced strongly and the density in the vena cava amounted to approximately 190 [DELTA]HU (Fig. 5C). Forty-five minutes after injection the densities in liver and spleen were 47 [DELTA]HU and 35 [DELTA]HU, respectively(data not shown). At this time point the density in the aorta had dropped to 51 [DELTA]HU (Fig. 5D).

Figures 5A-5D. Computed tomography blood-pool imaging in a rabbit with unmodified liposomes: (A) precontrast image, (B) 10 seconds after injection, (C) 15 seconds after injection (vena cava = approximately 190 [DELTA] Hounsfield units[HU]), (D) 45 minutes after injection (aorta= approximately 51 [DELTA]HU, liver = approximately 47 [DELTA]HU).

The DSPE-PEG liposomes showed a very similar imaging behavior compared with the unmodified liposomes with some delineation of the liver vasculature already after 28 seconds after injection (Fig. 6B). After 1 minute, the liver vessels were opacified brightly and the density in the aorta amounted to 125 [DELTA]HU (Fig. 6C). At 45 minutes after injection the density in the aorta had dropped to 71 [DELTA]HU (Fig. 6D).

Figures 6A-6D. Computed tomography blood-pool imaging in a rabbit with DSPE-PEG liposomes: (A) precontrast image (B) 28 seconds after injection, (C) 1 minute after injection (aorta = approximately 125 [DELTA] Hounsfield units [HU], vena cava = approximately 144 [DELTA]HU), (D) 45 minutes after injection (aorta = approximately 71 [DELTA]HU, vena cava = approximately 72 [DELTA]HU).

On the basis of the fitted curves of the aorta density differences obtained for free and liposomal iopromide(250 mg total iodine/kg body weight), areas under the curve (HU x minute) were calculated for different time periods (Fig. 7). As can be seen, vascular residence time increased in the following order: free iopromide << CHHS-PEG < unmodified <= DSPE-PEG. Interestingly, the areas under the curve obtained for the unmodified liposomes were as high as those obtained for the DSPE-PEG liposomes during the first 30 minutes after injection.

Figure 7. Areas under the curve (Hounsfield units x minutes) obtained for free and liposomal iopromide at a total iodine dose of 250 mg/kg body weight.

Discussion^

The potential of surface-modified contrast-carrying liposomes as blood-pool agents in MR imaging and nuclear medicine could be shown already.5 In the present study, the suitability of PEG-coated iopromide liposomes as a

CT blood-pool imaging agent was stu..d in comparison with unmodified lipo..nes for the first time.

In contrast to earlier studies with PEG-ylated liposomes, a new effective coating procedure was used here that involved simple mixing of preformed vesicles with either 5 mol% DSPE-PEG or CHHS-PEG overnight. The successful membrane incorporation of the PEG-derivatives is reflected in the drastic reduction of the zeta potential, which probably is the result of a shielding of SPG's negative charge (Table 1).10 Although encapsulation was not affected significantly by this procedure the mean diameter of the DSPE-PEG liposomes considerably rose to approximately 200 nm probably due to vesicle aggregation and fusion.13 As shown in Figure 2, the DSPE-PEG-containing liposomes displayed the highest stability in human plasma over the whole study period. This is consistent with steric stabilization of the liposomes, which prevents leakage of the encapsulated material due to reduced interactions with plasma proteins.4 After 6 hours, no significant difference in plasma stability existed between the unmodified and the CHHS-PEG liposomes, suggesting that removal of the polymer from the liposomes as has been found for collagen-coated small unilamellar vesicles.14 Most likely, CHHS is not as effective as DSPE in anchoring the PEG to the liposome membrane as has been found for CH-PEG.15

In the biodistribution study in rats (250 mg total iodine/kg body weight), no significant differences in blood concentration were found between the different formulations during the first hour (Fig. 3A). The resulting blood concentration of 1.2 mg iodine/g or 30% of dose (1 hour after injection) is not due to the unencapsulated iopromide that should have been removed almost completely from the intravascular space at this time point. In the case of the unmodified liposomes, the observed prolongation in circulation time is most likely due to a transient saturation of MPS-uptake (opsonisation or phagocytosis), which is known to occur at high lipid doses.3,16

Four hours after injection, the DSPE-PEG liposomes displayed the significantly highest blood level with approximately 1.1 mg iodine/g (28% of dose), which seems to be due to a more effective surface-modification, which was demonstrated in the plasma stability experiments.

Over the 24-hour study period, the resulting maximum liver concentration was well below 7% of dose (0.4 mg iodine/g) in all cases (Fig. 3B) demonstrating low MPS affinity of these vesicles. Surprisingly, the CHHS-PEG liposomes consistently showed the highest concentration in the liver suggesting a specific uptake mechanism.

In the spleen, a strong accumulation was found for the intermediate-sized (150 nm) CHHS-PEG liposomes 24 hours after injection amounting to 4.9% of dose while the larger DSPE-PEG liposomes (200 nm) displayed a distinct drop to 2.2% of dose at this time point. Based on a study on the size-specific spleen uptake of PE-PEG-coated liposomes, one would have expected a higher spleen accumulation of the latter (larger) liposomes.17

With regard to the application of iopromide liposomes for CT-imaging an orientating study (n = 1) was performed in rabbits using a total iodine dose of 250 mg/kg body weight. During the bolus phase, very high densities could be measured in the aorta (data not shown), which also are obtained after bolus application of larger doses of free contrast agents in CT angiography. In contrast to conventional contrast media, which are eliminated rapidly from the blood and only result in short-term blood-pool enhancement, the tested liposomal preparations showed prolonged blood opacification (Fig. 6). The unmodified as well as the DSPE-PEG liposomes displayed aorta density differences above 70 [DELTA]HU for up to 20 minutes (Fig. 4) showing similar imaging quality (Fig. 5 and 6). It can be assumed that an intravascular iodine concentration of >= 2 mg/mL (50-60 HU) over a period of at least 15 minutes should be sufficient for blood-pool imaging without the need for reinjection of the contrast agent. This would be useful especially for imaging of cardiovascular disease(eg, stenosis, ischemia, atherosclerosis), abnormal capillary permeability (eg, inflammation, cancer), and tumor neovascularity.18

The blood-pool enhancement that was obtained here(n = 1) is superior to that found in a CT-imaging study with an iodinated carboxymethyldextran derivative that allowed detection of partial ischemia in the rabbit liver.19 Until now, more effective blood-pool enhancement has been reported only for diatrizoic acid derivative nanoparticles(80 kV, 150 mA), which, however, displayed unpredictable pharmacokinetic and biodistribution properties.20

In conclusion, conventional and DSPE-PEG-coated iopromide liposomes were shown to be useful as blood-pool imaging agents for CT. Future studies are needed to demonstrate acceptable tolerance and elimination properties

as well as sufficient diagnostic potentia. in pathologic situations for these agent..

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KEY WORDS: Liposomes; iopromide; computed tomography; polyethylene glycol; biodistribution; blood-pool agent

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TAB F

INVESTIGATIVE RADIOLOGY
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Characterization of Continuously Extruded Iopromide-Carrying Liposomes for Computed Tomography Blood-Pool Imaging

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Leike JU, Sachse A, Rupp K. Characterization of continuously extruded iopromide-carrying liposomes for computed tomography blood-pool imaging. Invest Radiol 2001;36:303–308.

RATIONALE AND OBJECTIVES. **Contrast-carrying liposomes are potentially useful as computed tomography (CT) blood-pool agents. In the present study, preliminary safety, pharmacokinetics, and the CT imaging behavior of continuously extruded iopromide-carrying liposomes were studied.**

METHODS. **Iopromide liposomes were prepared by continuous high-pressure extrusion. Cell membrane–damaging characteristics were assessed in vitro in dog erythrocytes. Acute and subchronic toxicity and pharmacokinetics parameters were determined in rats. Computed tomography imaging efficiency was studied in rabbits.**

RESULTS. **The iopromide-carrying liposomes caused only minor morphological changes in dog erythrocytes. The median lethal dose in rats was approximately 4.5 g of total iodine per kilogram of body weight. In a subchronic tolerance study in rats that were administered six doses of 1 g iodine per kilogram twice a week, no adverse effects were observed. The pharmacokinetics in rats was dose dependent, and elimination of iopromide was almost complete within 7 days after intravenous administration. In rabbits, at a dose of 300 mg total iodine per kilogram, the iopromide-carrying liposomes displayed prolonged blood circulation, with mean CT density differences >60 Hounsfield units (aorta) for up to 10 minutes.**

CONCLUSIONS. **The iopromide liposomes were well tolerated, almost completely excreted, and have potential as a CT blood-pool imaging agent.**

KEY WORDS. **Liposomes; iopromide; tolerance; pharmacokinetics; elimination; computed tomography; blood-pool agent.**

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THE USE of liposomes as carriers for various agents in therapeutic, diagnostic, and preventive medicine has attracted much interest in recent years.[1] In the diagnostic field, liposomes have successfully been used experimentally as carriers for contrast agents in computed tomography (CT), magnetic resonance imaging, and nuclear medicine.[2,3] Owing to the rapid uptake of conventional contrast-carrying liposomes by cells of the mononuclear phagocytic system after intravenous injection, the use of such agents in radiology was mainly confined to liver and spleen imaging.[4]

In the past few years, however, surface-modified (mostly by the inclusion of lipid derivatives of polyethylene glycol in the liposome membrane) liposomes were shown to be able to avoid the mononuclear phagocytic system for prolonged periods, thus allowing drug targeting to non–mononuclear phagocytic system organs.[5] It has been suggested that such liposomes with extended lifetimes in the circulation might be useful as blood-pool (vascular) imaging agents, especially for magnetic resonance imaging and scintigraphy.[6] Recently, it has been demonstrated that at the high doses needed for CT, surface-modified as well as conventional iopromide-carrying liposomes (composed of soy phosphatidylcholine [SPC], cholesterol, and soy phosphatidylglycerol [SPG] in a molar ratio of 6:3:1) are potentially suited for blood-pool imaging.[7]

In the present study, tolerance, elimination, and diagnostic properties of the latter liposomes were studied. The results of different in vitro (influence on erythrocyte morphology) and in vivo (systemic tolerance and pharmacokinetics in rats and imaging efficacy in rabbits) studies are presented.

Materials and Methods

Preparation of Iopromide-Carrying Liposomes

Iopromide liposomes composed of SPC, cholesterol, and SPG in a molar ratio of 6:3:1 were prepared by the contin-

uous high-pressure extrusion method with the use of 0.4-μm filters for the last extrusion step.[8] The size of the resulting liposomes was measured by photon correlation spectroscopy with a Nicomp 270 submicron particle sizer (Nicomp Instruments Corp., Goleta, CA). The encapsulation efficiency was determined by equilibrium dialysis in a Dianorm system (Dianorm, Munich, Germany).

Erythrocyte Morphology

Approximate cell membrane–damaging characteristics of the liposomal formulation were assessed by investigating morphological changes in dog erythrocytes. Six parts by volume of fresh, heparinized whole dog blood (from one male and one female) were mixed with one part by volume of liposomal formulation (final iodine concentration 14.7 mg I/mL). Immediately and 1 and 2 hours after being mixed, the morphological changes in the erythrocytes were scored by observation under the light microscope (degree of damage 0–6).[9] Physiological (0.9%) saline solution served as a control.

In Vivo Characterization of Iopromide-Carrying Liposomes

The animal experiments were conducted in accordance with the guidelines of the German Animal Protection Law and were approved by the state agency supervising animal experimentation.

Systemic Tolerance After Single Intravenous Injection in the Rat

Rats (outbred animals of the Wistar strain, weighing 90–110 g; Schering SPF, Schering AG, Berlin, Germany) received increasing doses of iopromide liposomes (3, 4.5, and 6 g total I/kg). Equal numbers of male and female animals were used. The suspension was administered into a tail vein at a rate of 2 mL/min. For each dose level, three rats were studied. The animals were observed for 7 days after injection.

Systemic Tolerance After Repeated Intravenous Injection in the Rat

Six rats (Wistar strain, starting weight 90–110 g, three males and three females; Schering SPF) received six doses of 1 g of total iodine per kilogram at a rate of 2 mL/min twice a week (Monday and Friday). Mortality, general observations, and body weight gain were determined. Twenty-four hours before the animals were killed, they were housed in metabolic cages for urine sampling. Three days after the last dose was administered, the rats were killed. The animals were investigated histologically (liver, spleen, lung, and kidneys). The following parameters were determined in serum samples: glutamic-oxaloacetic transaminase, glutamic pyruvic transaminase, gamma glutamyl transferase (γ-GT), cholesterol, and blood urea nitrogen. In urine samples, γ-GT and lactate dehydrogenase were also determined.

Pharmacokinetics in Rats

The experiments were performed in four groups of male rats (Wistar strain, Schering SPF). The animals received either 250 or 1000 mg of total iodine per kilogram (low or high dose). The liposome suspension was administered into a tail vein at a rate of 2 mL/min. In two groups of rats (n = 4 per group, 286–320 g body weight), blood sampling from a catheter implanted into the carotid artery (0.4 mL per time point) was performed at 1, 5, 10, 20, 30, 60, 90, 120, 180, and 360 minutes after administration of the two different doses.

In the remaining groups of animals (n = 5 per group, 90–110 g body weight), urine and feces were collected up to 7 days after administration of the low or high dose. For the first 6 hours after drug administration, the animals were kept in metabolic glass containers to collect urine and feces. Then the rats were transferred to metabolic cages where they had free access to food and water. Urine was collected at 0.5, 1, 2, 3, 6, and 24 hours after administration and then daily for 7 days. Feces was collected daily for 7 days after administration.

At the end of each part of the study, the animals were killed and tissues and organs were removed for further study. An x-ray fluorescence analyzer (Prof. L. Kaufman, Department of Radiology, UCSF Medical School, San Francisco, CA) with an americium-241 source was used to determine the total iodine concentration in different matrixes.[10] The lower limit of quantification was determined to be 0.01 mg I/mL.

CT Study in Rabbits

Five male rabbits (New Zealand White strain) weighing 3.0 to 3.9 kg were obtained from a breeder (Schriever, Bremervörde, Germany). At the beginning of the imaging study, the animals were anesthetized by intramuscular administration of a mixture of xylazine (5 mg/kg) and ketamine (25 mg/kg). A mixture of the same drugs (33.3 mg ketamine/kg × h and 1.1 mg xylazine/kg × h diluted in physiological saline) was used intravenously (via an ear vein) to maintain anesthesia during the investigation. The animals were intubated and ventilated with room air. Before ventilation, spontaneous respiration was inhibited through intravenous administration of repeated doses of suxamethonium chloride.

The liposomes were injected manually as a bolus at a dose of 300 mg total iodine per kilogram via an ear vein. The rabbits were imaged by CT (PQ-2000, Picker, Cleveland, OH), with scans taken while the respirator was turned off. Precontrast and postcontrast (up to 60 minutes after the beginning of the application) helical 1.5- and 5-mm scans were obtained from the lungs to the urinary bladder of the animals by using the following settings: 130 kV, 100 mA, 14-cm field of view. Region-of-interest measurements for

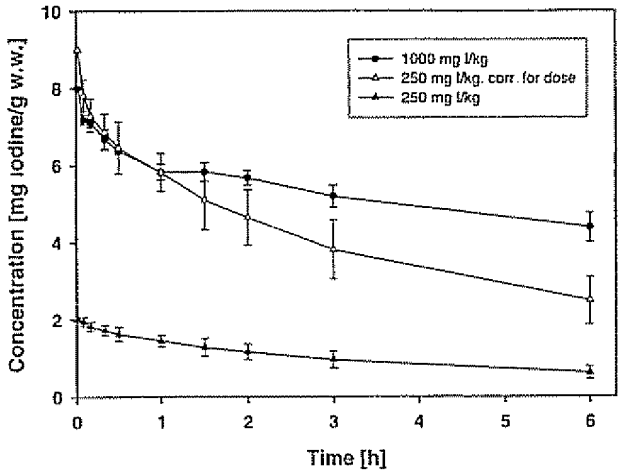

Figure 1. Blood level of iopromide in rats after intravenous injection of 250 or 1000 mg I/kg body weight iopromide liposomes. The data for 250 mg I/kg were corrected for dose (factor of 4). Mean ± standard deviation of four animals is given. w.w. indicates wet weight.

TABLE 1. Pharmacokinetic Parameters of Iopromide After Intravenous Injection of Iopromide-Carrying Liposomes to Groups of Four Rats at Doses of 250 and 1000 mg Total Iodine/kg

	250 mg I/kg	1000 mg I/kg
Blood parameters		
MRT (h)	6.55 ± 2.54	15.3 ± 1.35
AUC, 0–∞ (mg × h/mL)	11.2 ± 3.19	98.9 ± 11.3
AUC, 0–6 h (mg × h/mL)	6.67 ± 0.86	32.1 ± 1.55
AUC, t_{final}–∞ (mg × h/mL)	4.54 ± 2.50	69.9 ± 34.8
Cl (mL/min × kg)	0.40 ± 0.12	0.17 ± 0.02
$t_{1/2}\lambda_{z,b}$ (h)	4.59 ± 1.81	10.7 ± 0.93
V_{ss} (L/kg)	0.14 ± 0.02	0.16 ± 0.00
Elimination		
Urine (0–7 d) (% of dose)	92.1 ± 3.16	86.8 ± 0.90
Feces (0–7 d) (% of dose)	7.38 ± 0.67	7.47 ± 0.30
$t_{1/2}\lambda_{z,u1}$ (h)	0.48 ± 0.07	0.44 ± 0.05
$t_{1/2}\lambda_{z,u2}$ (h)	14.7 ± 3.03	29.3 ± 7.59

MRT indicates mean residence time; AUC: area under the curve; Cl: total blood clearance; $t_{1/2}\lambda_{z,b}$: elimination half-life, calculated from blood; V_{ss}: distribution volume at steady state; $t_{1/2}\lambda_{z,u1}$: half-life of renal excretion, first phase; $t_{1/2}\lambda_{z,u2}$: half-life of renal excretion, second phase.

Values are mean ± SD.

the scans were obtained from the aorta, caudal vena cava, liver, spleen, and kidney.

Data Analysis

Pharmacokinetics analysis was performed using the computer program Topfit (2.1).[11] The density differences obtained in the CT imaging study in various organs were listed as changes in Hounsfield units (ΔHU; postcontrast minus precontrast density).

Results

Liposome Properties

The iopromide-carrying liposomes used in this study were prepared by continuous high-pressure extrusion of a mixture of SPC/cholesterol/SPG at a 6:3:1 molar ratio as liposomal membrane components and were used without prior removal of the unencapsulated contrast agent. The resulting mean diameter amounted to 201 nm, and the mean encapsulation of iodine was 50%.

Erythrocyte Morphology

The iopromide-carrying liposomes caused only minor morphological changes in dog erythrocytes. The effect on the shape of erythrocytes was almost the same as the effect seen with the physiological (0.9%) saline solution (degree of damage at 2 hours after mixing was 0.31:0.25).

Systemic Tolerance After Single Intravenous Injection in the Rat

The approximate median lethal dose (LD_{50}) of the iopromide-carrying liposomes was 4.5 g total iodine per kilogram body weight in rats.

Systemic Tolerance After Repeated Intravenous Injection in the Rat

All animals survived the study. The body weight gain was not influenced by treatment. Three days after the last dose was given, a turbid, milky serum was observed. The clinical chemical parameters glutamic-oxaloacetic transaminase, glutamic pyruvic transaminase, γ-GT, and blood urea nitrogen in the serum were not affected. In the urine, a slight increase of γ-GT was measured. The lactate dehydrogenase in urine was not affected. Histological examination did not reveal any damage in the tested organs. However, a discernible foamy cell formation was observed in the spleen.

Pharmacokinetics in Rats

The blood concentration levels of iopromide after the low and high dose are illustrated in Figure 1. There was a clear dose dependence of pharmacokinetic parameters, especially of total clearance and terminal half-life (Table 1). Seven days after administration of the liposome suspension, almost complete elimination of the liposomal iopromide (measured as iodine) mainly via the urine was found. Only after application of the high dose (1000 mg I/kg) was a total of approximately 0.2% of the dose recovered in the liver and spleen. No residual iodine could be detected at this time point in the kidney, lung, blood, bone, and carcass.

CT Study in Rabbits

The CT study in rabbits indicated prolonged blood-pool opacification after bolus administration (within 15 seconds) of the iopromide liposomes. In general, a biphasic pattern was seen in the aorta. Peak enhancement was observed

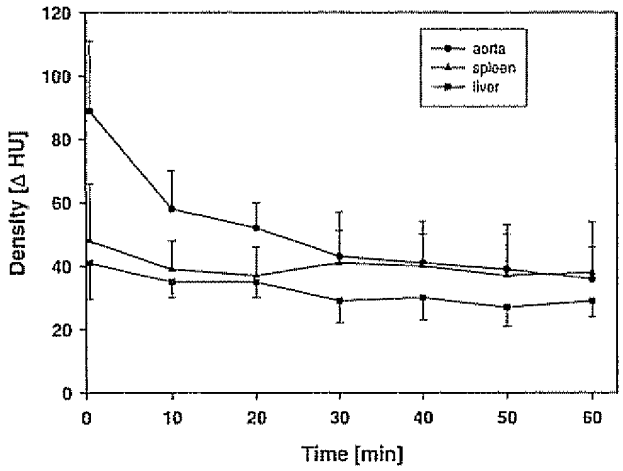

Figure 2. Time course of density difference (in changes in Hounsfield units [ΔHU]) in the aorta, liver, and spleen of rabbits after intravenous administration of 300 mg I/kg iopromide liposomes. The data represent mean ± standard deviation of five animals.

immediately after administration, followed by a plateau phase between 10 and 60 minutes (Fig. 2). Mean peak enhancement in the aorta was approximately 90 ΔHU. Ten minutes after administration, the mean blood density was approximately 60 ΔHU. At the same time point, the liver and spleen densities reached a plateau around 35 and 40 ΔHU, respectively. Figure 3 shows a selected pre- (Fig. 3A) and postcontrast (immediately after injection, Fig. 3B) image from a slice through the liver of a single animal.

Discussion

In a previous article, we had demonstrated for the first time the suitability of distearylphosphatidylethanolamine–polyethylene glycol–coated as well as unmodified iopromide liposomes for CT blood-pool imaging.[7] In the present study, tolerance, elimination, and diagnostic properties of unmodified (conventional) iopromide-carrying blood-pool liposomes were studied.

The in vitro test on the morphological changes in erythrocytes served as a convenient means of assessing membrane-damaging characteristics of this new contrast medium. To minimize disturbances in the microcirculation caused by rigidification of the erythrocytes, the use of contrast agents producing only minor changes in erythrocyte morphology is favored. The tested liposomes caused only negligible morphological changes in erythrocytes. Their effect an erythrocyte morphology was comparable with the effect of the control solution (physiological saline).

General tolerance was tested after both a single injection and repeated intravenous injections in rats. The approximate LD_{50} of the iopromide liposomes was 4.5 g total iodine per kilogram. In contrast to this, an LD_{50} of approximately 11 g I/ kg has been reported for nonliposomal iopromide in this

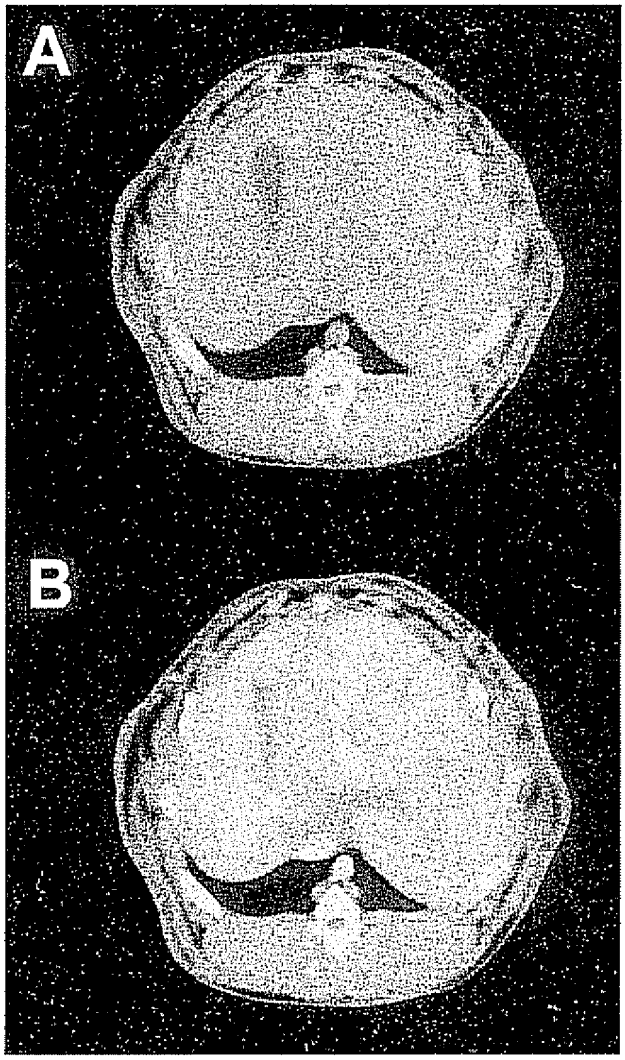

Figure 3. Computed tomography blood-pool imaging in a rabbit after intravenous administration of 300 mg I/kg iopromide liposomes. (A) Precontrast image. (B) Immediately postinjection.

animal species.[12] The difference in tolerance can partly be explained by the fact that in addition to the contrast medium, a high dose of lipid is given (initial iodine to lipid ratio = 1:1.5). The latter might also be responsible for the turbid appearance of the serum, which was observed in the repeated-dose toxicity study (six doses of 1 g I/kg, ie, 6 × 1.5 g lipid/kg). Furthermore, intracellular uptake of liposomal iopromide by the mononuclear phagocytic system most likely contributes to the increased toxicity. In the case of rat spleen, this uptake led to a discernible foamy cell formation in the red pulp after subacute dosing.

In rats, nonliposomal iopromide is eliminated mainly via the kidney. Approximately 90% of the dose is excreted with the urine and 10% with the feces.[13] Similar values were obtained in the present elimination study after administra-

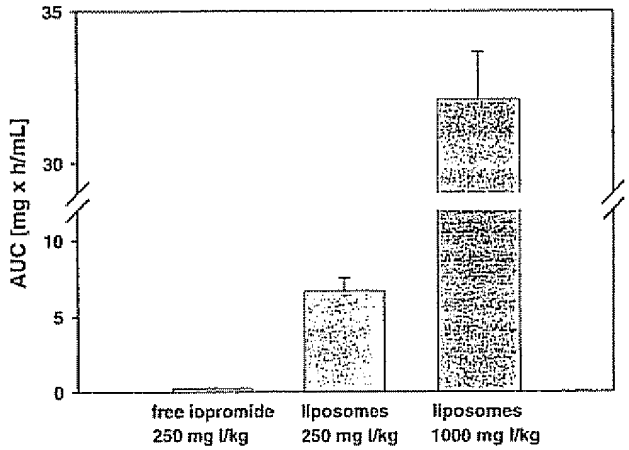

Figure 4. Areas under the curve (AUC 0–6 hours; mg × h/mL blood) of iopromide after intravenous injection of iopromide-carrying liposomes to groups of four rats at doses of 250 or 1000 mg total I/kg body weight. Mean ± standard deviation of four animals is given.

tion of iopromide-carrying liposomes. At doses of 250 and 1000 mg I/kg, almost complete excretion of the liposomal iopromide was found within 7 days after injection. The terminal half-life in blood is dose dependent and increased from 4.6 hours for a 250 mg I/kg dose to 10.7 hours for 1000 mg I/kg, suggesting a longer circulation of the liposomes after higher doses. The reason for this effect might be mainly the limited degradation capacity of the blood after administration of the extremely high dose of 1000 mg I/kg (ie, 1500 mg lipid/kg). Nonliposomal iopromide has a terminal half-life of approximately 0.3 hour.[13] Concomitant with this increase in half-life, the total clearance decreased from 0.40 to 0.17 mL/min × kg. The distinct difference in the area-under-the-curve 0–∞ value between the blood-pool liposomes and free iopromide is illustrated in Figure 4. The liposomes displayed a dramatically higher area-under-the-curve 0–∞ value compared with free iopromide at the same dose (250 mg I/kg). The pharmacokinetic properties of the small (mean diameter ≈200 nm) liposomes used in the present study show remarkable differences compared with the iopromide liposomes that we have tested as liver imaging agents in the past (mean diameter ≈470 nm; lipid composition phosphatidylcholine/cholesterol/stearic acid 4:5:1 molar ratio).[14] At the low dose of 250 mg I/kg, the terminal half-life obtained for the blood-pool liposomes is considerably longer (4.6 vs. 0.8 hours), and the clearance is lower (0.40 vs. 2.18 mL/min × kg), suggesting a prolonged avoidance of the mononuclear phagocytic system, which is partly due to the smaller size but, more important, due to the nature of the negatively charged lipid used (SPG instead of stearic acid).

The CT imaging properties of the iopromide liposomes were investigated in rabbits after intravenous injection of a total iodine dose of 300 mg/kg body weight. In contrast to conventional contrast media, which rapidly "leak" out of the vascular system, the tested liposomal formulation showed prolonged blood opacification (see Fig. 2). The liposomes

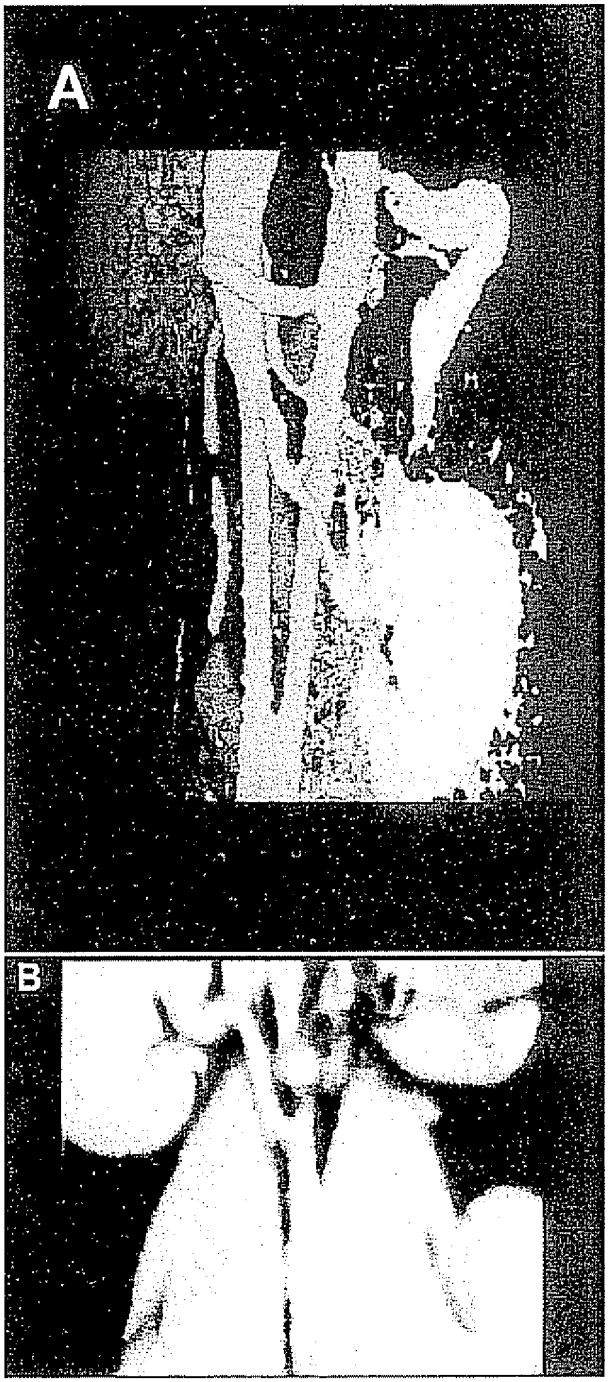

Figure 5. Computed tomography blood-pool imaging in a rabbit after intravenous administration of 300 mg I/kg iopromide liposomes. Vascular reconstruction of the abdominal region obtained immediately after administration of iopromide liposomes. (A) Three-dimensional reconstruction. (B) Multiplanar reconstruction.

displayed aorta density differences between approximately 90 ΔHU immediately after application of the liposomes and about 60 ΔHU at 10 minutes postinjection. The initial peak enhancement was probably caused by the sum of enhancements of intravascular liposomes and unencapsulated iopromide. The decline of enhancement up to 10 minutes was presumably mainly caused by the excretion of free iopromide. In previous CT studies in rabbits[7] and baboons[15] with similar doses of blood-pool liposomes, a somewhat higher blood enhancement was obtained. Owing to the moderate enhancement in this rabbit CT study, only in the first few minutes is sufficient blood-pool imaging possible, and vascular reconstructions (three dimensional, multiplanar reconstruction) can also be derived from the data (Figs. 5A and 5B). Owing to the good tolerance of the iopromide-carrying blood-pool liposomes, which was proved in systemic tolerance tests, additional CT studies at higher liposome doses should be carried out to increase the density differences in blood and to maximize the vessel-to-background contrast. Such prolonged blood-pool opacification could be especially useful for imaging of cardiovascular disease, abnormal capillary permeability (eg, inflammation, cancer), and tumor neovascularity.[16]

Until now, more effective blood-pool enhancement has only been reported for diatrizoic acid–derivative nanoparticles (80 kV, 150 mA), which, however, displayed unpredictable pharmacokinetic and biodistribution properties.[17] New CT techniques have distinctly shortened examination times and have therefore increased the utility of conventional contrast media for the diagnosis of vascular abnormalities. However, conventional contrast agents rapidly leak out of the vascular system, which leads to rapid dynamic distribution changes in different organs. Additionally, renal elimination of such agents results in a rapid decline in plasma concentration below the level that would be needed for angiographic investigations.[18] As shown, the present liposomes potentially overcome these limitations by remaining in the blood pool over prolonged periods. Furthermore, such liposomes can also be used as contrast agents for bolus dynamic CT imaging for both the liver and other abdominal organs (dual agents).[19] In addition, a distinct improvement in the delineation of liver lesions can be shown with the present liposomes over prolonged periods (authors' unpublished data, 2000).

In conclusion, the continuously extruded iopromide-carrying liposomes used in the present study are well tolerated, almost completely excreted, and useful for CT blood-pool imaging. Future studies are needed to optimize the dose as well as to demonstrate a sufficient diagnostic potential in pathological situations.

Acknowledgments

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